THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

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Medical Press

CXXX. RESPIRATORY QUOTIENT IN OBESE SUBJECTS.

By DAVID MURRAY LYON, DERRICK MELVILLE DUNLOP and CORBET PAGE STEWART.

From the Clinical Laboratory, the Royal Infirmary, Edinburgh.

(*Received April 2nd, 1932.*)

Introduction.

A long series of determinations of the B.M.R. in cases of obesity under treatment, accumulated over many months, showed that while the B.M.R. was generally within normal limits, the R.Q. was usually low, and often below 0·70. Quotients below 0·70 are by no means unknown in the work of Benedict on healthy subjects during fasting [*cf.* Benedict and Higgins, 1912]. Lehmann and his co-workers [1893], in the study of a fasting individual, observed low values of the R.Q. which, during several days, averaged 0·67. Bernstein and Falta [1916] obtained average quotients of 0·657 and 0·653 while feeding a normal individual on oatmeal, wheat, and butter; and on two different days quotients as low as 0·631 were recorded. Heinbecker [1931] has reported similarly low values in his study of the metabolism of Eskimos, and it is noteworthy that he specially remarks the relative absence of acetonuria in association with these abnormal values. The very low quotients encountered in diabetes, starvation or after administration of a ketogenic diet are undoubtedly due, in part at least, to ketosis, which means, in effect, the storage of oxygen. This is undoubtedly a factor in the low values reported by Means [1915]. Such an explanation of the results in our non-diabetic obese patients is, however, inadmissible, since the urine of these patients never contained more than a trace of ketones.

There is no doubt, however, that a value for the R.Q. below 0·69 in a diabetic subject, or 0·72 in a normal, is regarded with suspicion as due to faulty technique [*cf.* Lusk, 1928, p. 671], and we therefore considered it essential to institute a rigorous testing of the method by which our low values had been obtained.

Method.

The method used for the determination of the B.M.R. and R.Q. was the open method, using Haldane's face mask and Douglas bags, and analysing the collected expired air with the aid of the Haldane apparatus. This technique was employed simultaneously for the routine hospital work and for other metabolic investigations, in the course of which the very few low values of

the R.Q. found were associated with an unexpectedly low B.M.R., and could well be explained by a leak in the apparatus. In the case, however, of the obese patients on a sub-caloric diet, the very low R.Q. was encountered with a considerable degree of constancy, and was not associated with a reduced metabolic rate.

The points at which technical errors may enter into the determination of the R.Q. are:

(1) the fit of the mask,
(2) leakage from the bags,
(3) faulty analytical technique,
(4) the subject.

(1) *The fit of the mask.* The fitting of the mask was carefully carried out in every case, and tests were always made for leaks before and after the collection of expired air. The subjects co-operated intelligently, themselves pointing out any tendency to leakage between the cushion and the face. The obese person, moreover, has just that type of physiognomy to which a mask may be accurately fitted. Although there is thus no *a priori* reason for any error from this source being confined to obese people, in a series of 28 experiments the R.Q. was determined by means of a mouthpiece and noseclip, with which any leak is practically impossible. The average value was 0·014 higher than the value obtained from the same subjects, on the same occasions, with the mask. Nearly all the patients complained of greater discomfort from the mouthpiece and noseclip, and this in itself is probably sufficient to account for the slight discrepancy between the two methods—especially as the average B.M.R. was also 2·5 % higher with the mouthpiece than with the mask. In any case, a difference of 0·014 is insufficient to bring many of the low values of the R.Q. within the normal range.

(2) *Leakage from the Douglas bags.* The Douglas bags were filled with expired air from a spirometer, itself rigorously tested for leaks, samples being removed during the filling. Ten minutes later—the maximum time which ordinarily elapsed between their being filled and measured—samples were again removed, and still further samples were obtained after 30 minutes. On each occasion three samples were taken, and analysis was done in duplicate. The results showed that in 10 minutes there was insufficient leakage to cause appreciable fall in the R.Q., though in 30 minutes enough CO_2 had been lost to lower the R.Q. by 0·01 to 0·02. Evidently our procedure of emptying the bags within 10 minutes of their being filled was safe but must be rigorously adhered to. In confirmation of this was the fact that samples of expired air collected from the side-tube of the Douglas bag during the course of actual respiration experiments gave, on analysis, results practically identical with those from the same bag after standing for 10 minutes.

(3) *The analytical technique.* The accuracy of the analytical technique was proved by satisfactorily accurate determinations of the R.Q. of absolute alcohol

combusted in glass vessels. Of course, samples of the laboratory air were analysed each day, and no experimental work with the apparatus was undertaken until such analysis gave satisfactory results.

(4) *Errors due to the subject.* Errors in the observed R.Q. may, of course, be due to the subject not being in a strictly basal condition, though any such errors would probably tend to raise rather than to lower the R.Q. In all our subjects, however, the strictest precautions were taken to ensure basal conditions, and they were all well habituated to the technique before serious observations were started. Careful observations were made of the subject's respiratory movements before and during the experiment, and we are satisfied that in all the results recorded there was no error from alteration in the type of respiration during the collection of the sample.

The R.Q. of normal individuals.

Any systematic error, leading to a general lowering of all our values of R.Q. was unlikely in view of the results obtained in routine hospital determinations. These, however, were not from healthy individuals, and we therefore thought it desirable to compare our value for the R.Q. of normal healthy subjects with that given by other workers.

Seven normal healthy women students, accustomed to breathing through a mask, were chosen for this experiment. After an ordinary mixed supper in hospital at 7 p.m., the subject remained fasting and in bed from 9 p.m. until the test was made shortly after 8 a.m. the next morning. Two determinations of the B.M.R. were made on each subject (all were within normal limits) and four samples of air were analysed from each bag. The quadruplicate analyses agreed, and an average basal R.Q. of 0·797 was obtained over the whole series, the lowest individual value being 0·760.

Du Bois [1927] states that after ordinary mixed fare the normal basal R.Q. lies between 0·80 and 0·84, and an average quotient of 0·83 was calculated by Joslin [1923] from the results obtained in 236 out of 239 normal individuals in the Harris and Benedict series [1919]. On the other hand, a study of 61 readings in Benedict's famous Squad A [Benedict *et al.*, 1919] shows an average R.Q., when the subjects were on normal diet, of 0·795—a figure which is identical with our own. We may mention that all our subjects were under the strictest possible basal conditions at the time of the test. Under these circumstances we believe that our mean value for the R.Q. in normal subjects is correct, and does not indicate any general artificial lowering of our determinations such as would invalidate the results obtained from the obese subjects.

General treatment of the subjects.

On entering the ward for the investigation of metabolism, the subject was at first given what would normally be a maintenance diet of about 2500 Cals. Most of the subjects, however, had been accustomed to eating considerably

more than this, and even so were only maintaining their weight and not increasing it. To them, therefore, the supposedly maintenance diet was in reality sub-caloric, and they almost invariably lost weight on it. This diet was continued for some days during which repeated determinations of the basal B.M.R. and R.Q. were made, and the patient was thus habituated to the technical procedures.

The patient was then given a diet of, usually, 1000 Cals. This was normally made up of 66 g. of protein, 38 g. of fat, and 100 g. of carbohydrate, but occasionally the amount of carbohydrate was reduced with compensatory addition of fat. Finally, the effect of the sub-caloric diet was reinforced by administration of thyroid extract.

Throughout the whole of this time, along with frequent determinations of the B.M.R. and R.Q., and estimations of the urinary nitrogen, the presence or absence of acetonuria was noted daily. In a number of instances, when acetone was present, the total excretion of ketones was determined quantitatively, but was never found to exceed 0·5 g. daily.

In this note on the treatment of the subjects, as in the discussion which follows, we have included only those points relevant to our present purpose. A fuller account, from the therapeutic aspect, is given by Lyon and Dunlop [1932].

Results and discussion.

Table I gives the results of the determinations of the B.M.R. and R.Q. in a number of typical cases, and the total number of determinations has been summarised in Table II.

The average of 34 estimations of the R.Q. in obese persons receiving a diet of 2000 to 2500 Cals. is 0·755, which is significantly below the normal average. As has been pointed out, however, this diet, normally sufficient for maintenance, is actually sub-caloric for the obese subject who was losing weight on it.

When the patient was receiving a more definitely sub-caloric diet of 1000 to 1200 Cals. daily, the average basal R.Q. was still lower—0·721—but still just within the range of normal metabolism. Addition of thyroid extract made little difference to the average basal R.Q., but it is evident, from the fact that in the thyroid group relatively few values below 0·70 were obtained, that there is, in individual cases, a tendency for the R.Q. to rise when thyroid is administered (*cf.* records of individual patients in Table I). Indeed, many of the unduly low values, which depress the average of the thyroid group were probably obtained before the full effect of the hormone had appeared.

It is possible that in experiments of short duration the observed R.Q. bears no true relation to the oxidative metabolism actually proceeding, even though it accurately represents the gas exchange during the experimental period. Theoretically, this must be the case in an experiment of infinitesimally short duration, though in a long one of, say, 24 hours the R.Q. undoubtedly represents the net result of metabolism during that time. Hence it is necessary to enquire

Table I.

Case	Date	Diet, Cals.	Acetonuria	R.Q.	O_2 used per hour, litres	B.M.R.
Mrs A. S.	27. xi. 31	2700	–	0·77	20·85	+18
	1. xii. 31	.	–	0·78	20·08	+14
	3. xii. 31	1000	–	0·74	20·62	+18
	7. xii. 31	.	–	0·70	19·74	+11
	10. xii. 31	.	–	0·68	19·59	+10
	14. xii. 31	.	+	0·78	18·40	+ 5
	17. xii. 31	.	–	0·70	20·19	+13
	22. xii. 31	.	+	0·69	18·55	+ 4
	24. xii. 31	.	–	0·69	18·63	+ 5
	28. xii. 31	.	–	0·77	17·80	+ 3
	31. xii. 31	.	–	0·67	18·75	+ 5
	4. i. 32	.	–	0·69	20·25	+15
	8. i. 32	.	–	0·76	19·37	+11
	11. i. 32	.	+	0·73	20·31	+ 8
	14. i. 32	.	–	0·75	18·35	+ 6
	18. i. 32	.	–	0·69	18·01	+ 4
	21. i. 32	.	–	0·70	19·47	+13
	22. i. 32	.	Trace	0·74	18·16	+ 6
	25. i. 32	.	Trace	0·70	18·55	+ 9
	28. i. 32	.	–	0·72	18·65	+ 9
	29. i. 32	.	Trace	0·75	16·81	+ 3
	5. ii. 32	.	–	0·69	16·17	– 5
	8. ii. 32	.	–	0·74	17·45	+ 4
	13. ii. 32	.	–	0·68	17·56	+ 4
Mrs M.	5. xii. 30	1800	–	0·77	14·80	– 7
	8. xii. 30	2000	–	0·78	14·63	– 4
	14. i. 31	1000	–	0·74	14·47	–11
	21. i. 31	.	Trace	0·74	13·03	–11
	28. i. 31	1000 + thyroid	–	0·76	16·75	+15
	3. ii. 31	.	–	0·76	17·92	+18
	10. ii. 31	.	–	0·76	.	+15
	12. ii. 31	1000	–	0·68	18·32	+19
	13. ii. 31	.	–	0·66	17·83	+17
	14. ii. 31	.	–	0·64	16·10	+ 9
Mrs O.	27. ix. 28	1000	–	0·75	15·84	+14
	3. x. 28	2000	–	0·76	.	– 8
	10. x. 28	.	–	0·81	10·76	–19
	18. x. 28	800	–	0·74	13·30	– 1
	25. x. 28	1000	–	0·76	12·90	– 3
	31. x. 28	.	–	0·77	13·44	+ 1
Miss M.	12. viii. 29	2000	–	0·77	15·76	– 9
	30. viii. 29	1000	–	0·76	.	+ 4
	31. viii. 29	.	–	0·69	17·93	+ 4
	22. x. 29	.	+	0·68	18·65	+13
	10. ii. 30	.	–	0·67	13·98	– 4
	11. ii. 30	.	–	0·67	12·76	– 3
	18. ii. 30	1000 + thyroid	–	0·75	15·38	+13
	10. iv. 30	1000	–	0·67	18·03	– 4
	11. iv. 30	.	–	0·67	15·42	– 6
	18. v. 30	1000 + thyroid	–	0·75	.	+14

Table II.

Calorific value of diet	Number of determinations	Average R.Q.	Range	Number below 0·70	
					%
2000 and above*	34	0·755	0·67–0·86	3	9
1500–2000	30	0·722	0·65–0·86	11	36
1000–1200 standard	117	0·721	0·63–0·87	51	44
1000 low carbohydrate	15	0·678	0·63–0·76	12	80
1000–1200 + thyroid	70	0·727	0·66–0·84	14	20

* These diets were sub-caloric for the patients receiving them, and the patients were losing weight.

whether, in our experiments of 6 minutes, the observed data (granting their accuracy) can be accepted as expressing the oxidative metabolism during that period. Our experimental evidence bearing on this point falls into three categories. Firstly, as a routine measure, two successive independent determinations of the R.Q. were made at an interval of about 10 minutes, but without any attempt at regulating the interval exactly. Secondly, in a number of cases, the interval between the two determinations was exactly half an hour. Lastly, in a few cases, four independent determinations were made, one after the other, the patient wearing the mask throughout. These three procedures would expose any haphazard variations, or any accidental tendency in the routine treatment to hit off a possible periodicity in the R.Q. In the vast majority of the results the successive estimations of the R.Q. and of the oxygen consumption agreed very closely, and in only a very few cases was there any marked and unaccountable discrepancy. Since the agreement of duplicate or quadruplicate determinations was the rule, it could not be ascribed to coincidence, and, consequently, the 6-minute determinations may justifiably be regarded as giving a true expression of the metabolism at that time. Nevertheless, it must always be realised that reliance on the technique of indirect calorimetry is dangerous, and that our results, together with the interpretation to be placed on them, must fall if any technical error (which, however, we have failed to detect) can be shown to account for them.

Our discussion and tentative interpretation of our results is based on the supposition that individual determinations of the R.Q. are reliable, and that therefore the subjects were at times exhibiting an abnormal type of metabolism. (It is to be noted that patients varied in the results they yielded; some gave a low R.Q. more frequently than others, though none invariably gave results below 0·70.) The fact that the averages in Table II are so near the normal minimum interests us only as showing that, with a distribution similar to that of healthy individuals, many values must have been below 0·70—if the average is correct. Of that there is no reason to doubt in view of the value obtained for the average R.Q. in normal healthy subjects.

An R.Q. below 0·70 can only mean that oxygen is being used without production of a corresponding amount of carbon dioxide—*i.e.* for some incomplete oxidation. It has already been pointed out that the ketonuria occasionally observed was inadequate to account for the low values of the R.Q. even in the patients who showed it. It might be objected, however, that in the special case of the obese subject, ketosis might occur without corresponding ketonuria, the excess keto-acid being stored in the fatty tissues by virtue of preferential solubility in fat. According to this hypothesis, ketosis would occur during starvation without ketonuria but with a low R.Q., and after a carbohydrate-containing meal the stored ketones would be oxidised with production of an abnormally high R.Q. We have measured the R.Q. in our obese subjects at regular intervals for 4 hours after a meal, but have never found evidence of this occurring.

We have, moreover, shown by direct *in vitro* experiments that there is no preferential solubility of ketone substances in fat as compared with Ringer's solution or serum. In a typical experiment, Ringer-Locke solution containing 60 mg. of acetoacetic acid per 100 cc. (p_H 7·40) was shaken with an equal volume of olive oil for 90 minutes. The two layers were then separated by centrifuging, and acetoacetic acid was determined by Van Slyke's method in the aqueous layer and in the original solution. The results were: before shaking, 59·75 mg. acetoacetic acid per 100 cc.; after shaking, 60·50 mg. per 100 cc. A similar experiment with acetone (*a priori* more likely to show solubility in fat than sodium acetoacetate) gave a similar result: before shaking, 244 mg. acetone per 100 cc.; after shaking, 216 mg. per 100 cc.

The only other incomplete oxidation of which one can conceive as taking place in significant amount is the conversion of protein or fat into carbohydrate. Evidence from the urinary D/N ratio of 3·65 in severe diabetes and in depancreatised or phlorhidzinised dogs indicates that about 58 g. of carbohydrate may be formed from 100 g. of protein. This figure is derived from study of the fate of ingested protein, of which a small part of the nitrogen is lost in the faeces. When the body is metabolising its own protein exclusively, so that the whole of the nitrogen appears in the urine, this figure may be slightly altered. However, any error introduced by its use is a very small one. Even if, on this basis, it be supposed that all the protein metabolised is converted to carbohydrate which is stored, it appears that the R.Q. for the remainder is frequently below 0·70. This, in the absence of incomplete oxidation of fatty acid to keto-acids, and after allowance for the possible conversion of glycerol to glucose, strongly suggests the formation of carbohydrate from fatty acid.

The calculation to which the experimental data have been submitted is shown below, and in Table III are given the results for a number of abnormally low values of R.Q., all obtained in absence of ketonuria. The formulae apply to the usage of oxygen and production of CO_2 on the assumption that maximal production of carbohydrate from protein is taking place and that at least as much carbohydrate as this is being stored. Hence if, on solution of the equations, y is zero, the gain of carbohydrate to the body is equal to the production from protein, if y is negative, less than this amount is being stored, if y is positive, additional sugar is being produced and stored from fat. There is no assumption that carbohydrate is not being oxidised—merely that with abnormally low R.Q.'s it is being produced to a greater extent than it is being burnt. Since it is highly probable that some carbohydrate is always being oxidised, a zero value of y still indicates the probability of formation of sugar from fat, and even with positive values of y only the net gain, and not the total production of carbohydrate is calculable.

The values for the non-protein R.Q. in Table III are evidently well below 0·707, even when full allowance is made for maximal carbohydrate storage from protein. If further allowance is made for conversion of glycerol to

Table III.

Case	Date	Total R.Q.	For 1 hour, fasting: Litres O_2	Litres CO_2	g. urinary N_2	Non-protein R.Q. with normal protein oxidation	Assuming maximal carbohydrate from protein: Non-protein R.Q.	g. fat completely oxidised	g. fat converted to carbohydrate
J. McW.	14. v. 31	0·670	14·76	9·89	0·470	0·634	0·673	6·21	1·13
	22. v. 31	0·675	12·31	8·32	0·422	0·643	0·680	5·19	0·77
	8. vi. 31	0·650	13·56	8·82	0·247	0·631	0·651	5·80	1·77
V.	17. vii. 29	0·666	14·27	9·51	0·410	0·638	0 668	6·04	0·89
	19. vii. 29	0·656	14·31	9·39	0·415	0·624	0·652	5·95	1·58
	22. vii. 29	0·678	13·37	9·07	0·380	0·652	0·683	5·78	0·77
A. C.	29. vii. 29	0·668	19·48	13·00	0·450	0·645	0·670	8·42	1·68
	1. viii. 29	0·650	20·28	13·20	0·465	0·626	0·652	8·55	2·56
	20. viii. 29	0·679	19·79	13·42	0·445	0·653	0·683	8·72	1·23
M.	10. ii. 30	0·670	13·98	9·37	0·260	0·653	0·672	6·17	1·14
	11. ii. 30	0·670	12·76	8·55	0·245	0·652	0·672	5·62	1·04
J. H.	12. iii. 30	0·677	17·24	11·67	0·510	0·649	0·683	7·41	1·04
Mn.	12. ii. 31	0·680	20·13	13·67	0·448	0·659	0·682	8·90	1·19
	13. ii. 31	0·665	19·49	12·95	0·482	0·640	0·667	8·35	1·80
	14. ii. 31	0·641	17·22	11·04	0·408	0·614	0·641	7·12	2·54
H.	12. ii. 31	0·683	18·32	12·51	0·448	0·662	0·687	8·09	0·88
	13. ii. 31	0·664	17·83	11·84	0·430	0·640	0·666	7·64	1·67
H. K.	17. ii. 31	0·696	15·81	11·00	0·650	0 661	0·704	6·73	0·07
	25. ii. 31	0·664	12·14	8·06	0·479	0·620	0·632	4·67	1·06
	5. iii. 31	0·700	12·47	8·73	0·576	0·660	0·711	5·25	0·00
E. S.	9. iii. 31	0·682	17·51	11·94	0·684	0·644	0·688	7·34	0·79
	10 iii. 31	0·680	16·22	11·02	0·690	0·636	0·685	6·68	0·79
	19. iii. 31	0·627	15·52	9·74	0·450	0·590	0·625	6·14	2·76
	27. iii. 31	0·630	16·22	10·21	0·390	0·601	0·628	6·56	2·71
A. S.	31. xii. 31	0·670	18·75	12·56	0·304	0·655	0·671	8·33	1·58
	18. i. 32	0·690	18·01	12·42	0·299	0·677	0·693	8·25	0·67
	13. ii. 32	0·680	17·56	11·94	0·320	0·665	0·683	7·87	1·04

Example of calculation.

I. *Data.*

(*a*) The figures given by Lusk [1928, p. 69] have been used for the consumption of oxygen and production of carbon dioxide during the normal oxidation of fat and carbohydrate:

1 g. fat requires 2·019 litres O_2 and gives 1·427 litres CO_2.
0·9 g. glucose, or 1 g. glycogen, requires 0·828 litres O_2 and gives 0·828 litres CO_2.

(*b*) In dealing with protein, Lusk's figures for the elementary composition of meat protein have been used, but it has been assumed that as body protein is being metabolised there is no loss in the faeces. This assumption may not be correct, but if anything it weighs the balance against the conclusion of fat conversion to carbohydrate, since it increases slightly the amount of protein carbon available for similar conversion.

	C g.	H g.	O g.	N g.
100 g. meat protein contain	52·88	7·27	22·68	16·65
Of this there appear in the urine	9·41	2·66	14·10	16·65
For respiration	43·17	4·61	8·58	—
Deduct water equivalent	—	1·07	8·58	—
	43·17	3·54	—	—

Hence 100 g. protein require 100·4 litres O_2 and give 80·6 litres CO_2.
Thus with normal metabolism,

1 g. urinary N≡6·005 g. protein≡6·025 litres O_2 used≡4·84 litres CO_2 produced.

With maximal conversion of protein to carbohydrate,

1 g. urinary N=3·65 g. glucose, which, on oxidation, would use 2·72 litres O_2 and give 2·72 litres CO_2.

Hence, with this glucose stored,

1 g. urinary N=3·305 litres O_2 used, and 2·12 litres CO_2 produced.

(R.Q. = 0·641.)

(c) For the hypothetical conversion of fat into carbohydrate, the fat is taken as having the composition of glyceryl dioleate monostearate, $C_{57}H_{106}O_6$, which represents fairly closely the average composition of body fat. Small differences in composition have, however, no appreciable effect on the result. It is further assumed that all the carbon of the fat molecule is retained in the carbohydrate molecule, the change being represented as:

$$2C_{57}H_{106}O_6 + 47O_2 = 19C_6H_{10}O_5 + 11H_2O.$$

According to this equation:

1 g. fat requires 0·594 litres O_2, produces no CO_2, and yields 1·736 g. of glycogen.

II. *Formulae.*

From these data, formulae are derived as follows:

Let observed O_2 usage over a given period $= a$ litres
" CO_2 production over a given period $= b$ litres
" urinary nitrogen over the same period $= c$ g.

With maximal carbohydrate production from protein,

O_2 for non-protein metabolism $= a - 3{\cdot}305\,c$.
CO_2 from non-protein metabolism $= b - 2{\cdot}12\,c$.

Let x g. fat be oxidised completely,
y g. fat be converted to carbohydrate.

Then O_2 used $= 2{\cdot}019x + 0{\cdot}594y = a - 3{\cdot}305\,c$.
CO_2 produced $= 1{\cdot}43x = b - 2{\cdot}12\,c$.

Whence
$$x = \frac{b - 2{\cdot}12\,c}{1{\cdot}43}.$$
$$y = \frac{a - 1{\cdot}41\,b - 0{\cdot}31\,c}{0{\cdot}594}.$$

carbohydrate, the non-protein R.Q. may be as low as 0·695, the fatty acid being completely oxidised. Values below this figure must then indicate incomplete combustion of fatty acid. The figures given in the last two columns of Table III represent the amounts of fat oxidised and stored as carbohydrate, on the assumption that such a conversion takes place, and that its equation is that postulated by Zuntz. Other calculations, based on other hypothetical equations, such as that of Chauveau, give higher figures for the amount of fat converted to carbohydrate, since they involve the simultaneous production of a little CO_2, and, in the case of Chauveau's equation, an R.Q. of 0·29 for the conversion. No stress is laid on the figures given in these two columns; they are merely intended to show that if conversion of fat into carbohydrate is taking place, it is proceeding to an appreciable extent, and to emphasise the difference between the observed R.Q. and the minimal value possible on the assumption of normal and complete oxidation of fatty acid.

On the assumption of carbohydrate formation from fat with conservation of all the carbon, Table III shows that the glucose production accounts for about 1/10 to 1/5 of the fat metabolised. These amounts are appreciable, but even if they be added to the amounts supposed to be formed from protein, they would not cause a very rapid increase in the glycogen reserves, and could therefore be conceived of as continuing for some hours.

It is obvious that such a storage of carbohydrate cannot continue indefinitely. Accordingly, it is found that after a meal, with the subject still resting, the R.Q. rises above 0·70, sufficiently to indicate usage of carbohydrate during this period. This indicates that the carbohydrate storage mechanism, if it exists at all, is essentially one called into action only during a fast. It is also noteworthy, that even with the patient continuously on the same sub-

caloric diet, the fasting R.Q. is not invariably below 0·70. There is a tendency for the R.Q. to rise and fall in a periodic way, as if the need for storage appeared when the carbohydrate reserve had fallen below a certain level, and disappeared when, by storage from food or synthesis, the reserve had been built up to some higher level.

If, however, it be objected that the actual storage of carbohydrate is so improbable that the assumption of its occurrence must be rejected, we are faced with still greater difficulties, for then we must assume that protein is being oxidised with its normal R.Q. of 0·806. This, of course, leads to still lower values for the non-protein R.Q.—values sometimes below 0·60—which cannot possibly be explained on any known hypothesis.

Without going into the whole of the vexed and still controversial question of the possibility of carbohydrate formation from fat—a question well reviewed by Rapport [1930]—we may mention a few points bearing directly on our interpretation of our experimental data. The obese person whose reserves consist predominantly of fat is evidently more likely than the normal individual to exhibit the phenomenon of fat conversion into carbohydrate under sub-caloric dietary conditions. Moreover, if we reject arguments in favour of such a conversion in diabetes based on observations of abnormally high D/N ratios, there is evidence that exogenous fat can be turned into carbohydrate in animals whose carbohydrate stores have been depleted by a period of starvation, and in which, therefore, the conditions are analogous to those obtaining in our patients. Similarly, Jost [1931] has recently observed increased sugar formation on perfusion of glycogen-poor livers with fatty acids and phosphate. It is significant, too, that in our experiments there was a tendency for a low R.Q. to appear more readily the smaller the amount of carbohydrate in the diet. The absence of ketonuria in our experiments may be reasonably explained by the assumption that part of the fat was oxidised *via* carbohydrate, thus affording a sufficiency of ketolytic material. Such a mechanism may also be the explanation of the fact, known from clinical observation, that fat diabetic subjects show less tendency to ketosis than thin ones.

In conclusion, therefore, we believe that our experimental results afford evidence that in the undernourished obese subject, fat is converted into carbohydrate. This conclusion is, however, tentative, inasmuch as it depends on a technique open to criticism, and becomes liable to revision if and when any more probable mechanism for the storage of oxygen is suggested. Valuable confirmatory evidence of the correctness of our hypothesis might be obtained from measurement of the actual heat production simultaneously with the respiratory exchange. Such a measurement would enable the calculated heat production to be compared with the actual. This technique was, however, beyond our means. Our interpretation does not of course imply that a conversion of fat into carbohydrate takes place ordinarily as an obligate step in the metabolism of fat; but simply that in certain abnormal circumstances the animal body is capable of it. Indeed, experiments on muscular exercise

[*cf.* Stewart, Gaddie, and Dunlop, 1931] indicate that when fat is used as a source of energy for external work, it is directly oxidised and is not first converted into sugar. In other words, the auxiliary verb for the conversion of fat to carbohydrate is, at most, "may" and not "must."

Summary.

1. In a series of 34 observations on the R.Q. of obese subjects on a subcaloric diet of 2000 or more Calories loss of weight occurred and the average R.Q. was 0·755. Of these, 3 were below 0·70.

2. When the diet was reduced to 1000 to 1200 Calories the average R.Q. in 117 observations was 0·721 with 51 below 0·70.

3. When the carbohydrate content of these latter diets was reduced, but the total calorific value maintained, the average R.Q. fell to 0·678, 12 out of 15 being below 0·70.

4. Administration of thyroid to the subjects on 1000 to 1200 Calories diet produced a slight increase in the R.Q., the average of 70 determinations being 0·727, with only 14 below 0·70.

5. Ketonuria was usually absent when these observations were made and when present was only slight.

6. These results are discussed with respect to the conversion of fat to carbohydrate.

The thanks of two of us (D. M. D. and C. P. S.) are due to the Medical Research Council for part-time grants.

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CXXXI. THE REACTION OF FISH-LIVER OILS WITH ANTIMONY TRICHLORIDE.

(PRELIMINARY COMMUNICATION.)

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(*Received June 7th, 1932.*)

It is a well-known fact that fish-liver oils and other products containing vitamin A give a blue colour reaction with antimony trichloride, the intensity of which, within certain limits and under definite conditions regarding the concentration of the solutions, runs parallel with the biological activity of the oil. As yet the question whether the substance reacting with $SbCl_3$ is the vitamin itself or an accompanying compound has not been definitely settled.

The purpose of the present communication is to report some experiments bearing on this problem.

It is difficult to obtain the blue product resulting from the interaction of antimony trichloride and liver oil in quantity and condition suitable for examination. As is well known, the blue colour is exceedingly unstable. It reaches its maximum intensity some few seconds after the mixing of the solution of oil in dry chloroform with the reagent (a saturated solution of antimony trichloride in chloroform). After about 60 seconds the colour has already begun to fade, and it disappears completely in the course of the next 15 minutes.

A number of other dry, inorganic salts[1] were tried with no evident advantage. With concentrates of vitamin A, a blue or bluish colour was always obtained, but the colour was no more stable and often fainter than that produced by the antimony trichloride. This happened especially with salts only slightly soluble in chloroform. As is well known, the addition of water makes the colour disappear almost instantaneously; the formation of the blue colour is also prevented by acetone, ether, acetaldehyde and various alcohols. The stability of the blue colour is largely influenced by even small temperature changes, an increase of 10° above the ordinary room temperature makes the colour fade more quickly, while a corresponding fall in temperature has the opposite effect. An intense cooling increased the stability of the blue colour for a considerable time. At − 35° it persisted for at least some hours, and after subsequent cooling to − 50° a heavy blue oil was precipitated on the bottom of the vessel.

[1] $AlCl_3$, $AlBr_3$, $AsCl_3$, $BiCl_3$, $FeCl_3$, $SbBr_3$, $SbCl_5$, $SnCl_4$, $SnBr_4$, $TeCl_4$, $TiCl_4$, $ZnCl_2$, $ZnBr_2$

It was therefore decided to work at a low temperature, and then to decompose the blue oil produced according to the following hypothetical equation.

1. Chromogenic substance (Vitamin A?) + $SbCl_3$ = Blue product.
2. Blue product + H_2O = Chromogenic fraction + SbOCl + 2HCl.

Liver oil of halibut was chosen as a convenient raw material. It contains 50–100 times more vitamin A than cod-liver oil, and at the same time it is relatively poor in vitamin D. The saponification was carried out in the usual way, the oil being boiled with an excess of 5 % alcoholic KOH. The saponification was repeated until a sample of the product was practically free from glycerides. The product was next dissolved in methyl alcohol, cooled down to about − 5° and the crystalline cholesterol filtered off. After evaporation of the methyl alcohol the residue consisted of a reddish-yellow oil, which showed an antimony trichloride value of 13,500 blue units (Lovibond)[1], and when tested on rats proved active in daily doses of 0·002–0·003 mg. All experiments were carried out in an atmosphere of nitrogen or carbon dioxide. The distillations were likewise performed in indifferent gases or *in vacuo* and at a low temperature.

Preparation of the blue product.

15 g. of the unsaponifiable fraction were dissolved in 600 cc. of dry chloroform, and the solution was at once cooled down to − 35° in a cooling mixture of carbon dioxide, alcohol and glycol. About 1300 cc. of a saturated solution of $SbCl_3$ in dry chloroform were added under constant stirring, care being taken that the temperature did not exceed − 30°. On subsequent cooling to − 55° a heavy blue oil was precipitated, from which the faintly coloured chloroform solution could be decanted. To this oil pieces of ice or an ice-cold mixture of water and 10 % KOH (for the neutralisation of the possibly harmful hydrochloric acid) were added under constant stirring until the blue colour disappeared. Then the mixture was extracted with benzene. The benzene solution was filtered, washed with water and subsequently dried with sodium sulphate and the benzene distilled off *in vacuo*. The residue weighed 11·25 g. It was a highly unsaturated reddish-yellow oil, which we may call "the chromogenic fraction," as it still reacts with antimony trichloride. The antimony trichloride value is however only 4000–5000 blue units, *i.e.* about 1/3 of the colour value given by the original concentrate. In doses of 0·01–1 mg. per day it showed no biological effect (23 rats). On the addition of ethyl alcohol to the oil an amorphous yellow powder was precipitated, the blue value ($SbCl_3$) of which was variable, *viz.* 1700–8700 Lovibond units. The reason apparently was that the powder, when dried and exposed to the air, underwent a rapid transformation and became almost colourless. The freshly prepared product had an unsharp melting-point between 160°–170°. It was easily soluble in benzene,

[1] Measured with the Rosenheim-Schuster colorimeter according to the method described by Rosenheim [1927], the highly active preparations being taken into consideration and the readings being made half a minute after the addition of the $SbCl_3$ solution.

chloroform and ether with an intense yellow colour. In daily doses of 0·002–0·02 mg. it was biologically inactive (24 rats). When the chromogenic fraction was treated with $SbCl_3$ at low temperature and the reaction product decomposed with water a chromogenic substance was again formed.

The non-chromogenic fraction.

On addition of cold light petroleum ($-45°$) to the faintly coloured chloroform solution mentioned above, the last traces of the blue product were precipitated. To the separated solution which was almost colourless, ice or cold aqueous KOH was added under constant stirring. The aqueous layer was removed in a separating funnel, the chloroform-light petroleum solution was dried with sodium sulphate and evaporated *in vacuo* on a water-bath. The residue, a faintly yellow oil, only gave a slight coloration with antimony trichloride and in daily doses of 0·05 and 0·4 mg. the biological effect was nil (6 rats).

The results of the biological experiments seem to justify the conclusion that the antimony trichloride reaction is not specific for vitamin A. This is in agreement with Euler and Euler [1930]. It was shown by these authors that a series of carotenoids devoid of any biological effect (vitamin A), gave a blue colour with antimony trichloride. In addition to its biological action, vitamin A, according to several investigators, is characterised by a broad, selective absorption in the ultra-violet region of the spectrum, with a definite maximum at 328 $m\mu$. It was accordingly desirable to make a spectrographic comparison of the chromogenic fraction and the liver oil concentrates of *Hippoglossus hippoglossus*. It was found that the exceedingly active preparations of the halibut-liver oil showed a very distinct absorption band in the ultra-violet range at 326–327 $m\mu$. This band was not observed in the chromogenic fraction. The characteristics of the absorption spectrum of this fraction seem to be the following.

A strong absorption in the ultra-violet from 390 $m\mu$ towards shorter wavelengths, with bands at 370, 351 and 300–290 $m\mu$. The absorption curves obtained with some of the preparations are presented in Fig. 1 and Fig. 2.

The absorption spectra given by the two blue products ($SbCl_3$ + concentrate, or chromogenic fraction) were compared both spectrographically and spectrophotometrically. As the spectrophotometric measurements in the Albrecht apparatus gave only approximate values, the more exact spectrographic data are alone reported. The spectrogram of the blue product, obtained from the concentrates, showed two absorption bands, at 622·5 (621·1) and 590 $m\mu$ respectively. The first was very sharp and its position easy to place, the other was less distinct and the wave-length in this case could hardly be determined with any degree of accuracy. In some preparations of the blue product the second band was localised at 575 $m\mu$. In contrast with the concentrate the chromogenic fraction is partially insoluble in alcohol. The blue product obtained from the chromogenic fraction exhibits two absorption bands, differing from those

seen in the blue product from the concentrate. In the case of the chromogenic fraction the maximum absorption is at 590 (586·8) $m\mu$ and 550–522 $m\mu$. Concerning these bands it is likewise to be noted that the first is the most

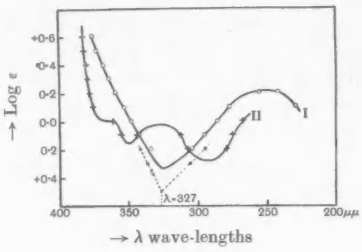

Fig. 1.

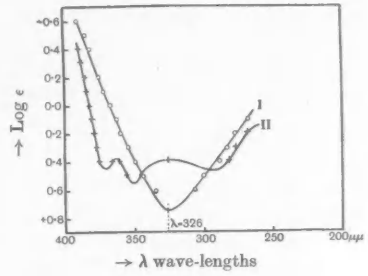

Fig. 2.

Absorption curves for the concentrate (I) and the "chromogenic fraction" (II).
The positions of the points marked • were determined photometrically, the others visually.
$\epsilon = 1/d \log I_0/I$.
I_0 = intensity of the incident light.
I = intensity of the transmitted light.
d = thickness of the cell in cm.

distinct. The positions of some of the absorption bands in the spectrograms mentioned above were determined both visually and photometrically. The values obtained with the photometer are given in parentheses.

The maxima of the most distinct bands can be localised with an accuracy of about ± 1–2 $m\mu$; as to the weaker bands the position cannot be determined with any great degree of accuracy owing to their lack of sharpness.

Discussion

The spectrographic results obtained with the vitamin A concentrates (of halibut-liver oil) are in close agreement with the findings of Morton, Heilbron and Thompson [1931], Heilbron, Gillam and Morton [1931], and Coward, Dyer, Morton and Gaddum [1931]. According to several investigators, the ultra-violet absorption with its maximum near 328 $m\mu$ has been observed in all the biologically active preparations examined, and seems to be characteristic for vitamin A. By treating the vitamin concentrates with $SbCl_3$ and adding water to the blue compound thus formed, the vitamin is rendered inactive.

The addition of water results in the formation of a yellow oil, which I have called the "chromogenic fraction." With antimony trichloride this fraction gives a strong blue colour, which cannot be attributed to vitamin A itself, but must be due to yet another chromogen. As mentioned by Heilbron, Gillam and Morton [1931] the concentrate of halibut-liver oil probably contains two chromogens.

One of these chromogens is supposed to cause the absorption at 328 $m\mu$, and that at 583 $m\mu$ of the blue product, while the other chromogen in the colour reaction is responsible for the absorption band at 620 $m\mu$. The physico-chemical characteristics of vitamin A, according to these authors, are as follows: "a colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near 328 $m\mu$ and giving with antimony trichloride a coloured substance, characterised by a maximum in the yellow between 580 and 590 $m\mu$, whilst the molecular extinction coefficients at 328 and 580–590 $m\mu$ expressed in terms of vitamin concentration should be approximately equal."

Comparing my own results with the findings of Heilbron, Gillam and Morton [1931], it is striking that my highly active concentrates exhibit with antimony trichloride a decided absorption maximum at 622 $m\mu$, while, on the other hand, the inactive preparation ("chromogenic fraction") in the colour test does not show this band, but exhibits a strong absorption in the neighbourhood of 590 $m\mu$ and a fainter one at 551 in the green part of the spectrum.

While Heilbron, Gillam and Morton state that oxidation of certain liver oils, containing vitamin A, leads to an increase in the intensity of the absorption at 606, or 620 for concentrates, I have observed that a destruction of vitamin A subsequent to treatment with antimony trichloride and water abolishes this band (622). On the other hand, the absorption at 590 $m\mu$ is exceedingly pronounced. I must conclude therefore that an absorption band at 590 $m\mu$ produced by the interaction of antimony trichloride and the "chromogenic fraction" cannot be attributed to vitamin A. It must however be taken into account that so powerful an influence on the inner structure of the substance, as a treatment with $SbCl_3$ and water must probably exert, may also show itself in a displacement of the absorption bands. From that point of view it is possible that the chromogenic nature of the concentrates after the treatment with $SbCl_3$ and water may be due to the decomposed vitamin.

Summary.

A certain parallelism exists between the biological activity and the intensity of the blue colour given by liver oil concentrates of *Hippoglossus hippoglossus* with antimony trichloride.

At room temperature the blue colour thus formed is exceedingly evanescent, but at $-40°$ the reaction-product is stable for hours.

Concentrates of halibut-liver oil probably contain two chromogens, of which one is possibly identical with vitamin A, and is destroyed by treatment with antimony trichloride and water, while the other, which presumably has no connection with growth in animals, still reacts with antimony trichloride forming a blue product from which the chromogen may be regenerated by the addition of water. The absorption bands of the liver oil concentrate and the "chromogenic fraction," together with the spectra of their reaction products with antimony trichloride, are described.

I am indebted to Professor E. Poulsson for his generous help and advice during the work and I also wish to thank Professor L. Vegard and Professor E. Langfeldt of Oslo University who kindly placed at my disposal the necessary instruments for measuring the absorption spectra.

Miss Qviller and Mr Stensholt gave me their valuable assistance during some of the spectrographic measurements.

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CXXXII. AN IMPROVED AND SIMPLIFIED BENZIDINE TEST FOR BLOOD IN URINE AND OTHER CLINICAL MATERIAL.

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(*Received June 8th, 1932.*)

THE principle of the benzidine test for blood, as is well known, depends upon the peroxidase action of haematin, the benzidine being oxidised to a blue compound. The great drawbacks to the test are the instability of the hydrogen peroxide solution and its impurities. Ozonic ether is often substituted for hydrogen peroxide on account of its greater stability but of course it is much more expensive. Theoretically hydrogen peroxide is the perfect oxidising agent, but the instability of aqueous solutions, together with the impurities—acids, sulphates, phosphates, and alkali metals—which are generally present, detract considerably from its usefulness in practical laboratory work, and blank tests are always necessary to control results.

About 24 years ago Tanatar [1908] produced a solid compound of hydrogen peroxide and urea which was stable, neutral and ashless, and free from inorganic impurities, and under the registered name of "Hyperol" it is available on the market at a low price. It contains 35 % of hydrogen peroxide, remarkably pure—the only impurity being the merest trace of citric acid which, however, is insufficient to affect titrations with methyl orange—and perfectly stable. It only begins to decompose when heated to 60°, and the decomposition results in the formation of oxygen, water and urea.

The stability, purity and strength of hyperol render it a most useful, reliable and powerful oxidising agent. As a powder it is easy and convenient to handle and will keep indefinitely provided it is not brought into contact with moisture. Containing 35 % of hydrogen peroxide, it is much more powerful than the 10 volume (3 %) aqueous solution which is generally used in laboratories.

In the benzidine test for blood hyperol can be used, instead of the hydrogen peroxide solution or ozonic ether, with considerable saving of expense, rendering the test much more delicate and reliable, and so simplifying the technique that in most cases it can be done as a "spot" test.

Method as applied to urine.

1. Place a small amount of benzidine (enough to cover the tip of a pocket-knife blade) on a clean white plate.
2. Add two drops of glacial acetic acid.
3. Add one drop of urine.
4. Add a small amount of hyperol (enough to cover the tip of a pocket-knife blade).

A bright deep blue colour appears immediately if blood be present, even in a very minute trace.

Sources of error. Urine containing potassium iodide, pus or enzymes will react to this test in the same way as with the ordinary guaiacum or benzidine tests. If pus or enzymes be suspected the urine should first be heated to over 60° (boiling is not necessary) and cooled before the test is applied. No reaction with pus or enzymes will be obtained under these circumstances.

If potassium iodide be suspected add a drop of dilute sulphuric acid (10–25 %) to the blue mixture when the blue colour will disappear. Next add a drop of fresh 1 % starch solution and the blue colour will reappear if due to iodides but will fail to reappear if due to blood.

Another and simpler method of detecting iodides is to place a drop of the urine on the white plate, without any benzidine, and add a small quantity of hyperol and then a drop of 1 % starch solution. A blue colour will result.

Marked alkalinity or acidity has practically no effect on the hyperol test whereas marked alkalinity prevents the development of the reaction for blood in the guaiacum test.

Relative sensitivity of guaiacum, ordinary benzidine and hyperol-benzidine tests for blood in urine.

From a large series of tests the following results were obtained:

	Guaiacum	Ordinary benzidine	Hyperol-benzidine
Smallest quantity of blood detectable	From 1 part in 1000 to 1 in 10,000	From 1 part in 25,000 to 1 in 60,000	From 1 part in 200,000 to 1 in 260,000

Thus with the hyperol-benzidine test described one can be sure of detecting 1 part of blood in 200,000 parts of urine as compared with the 1 part in 1000 revealed with the guaiacum test. It is of course much more sensitive than the spectroscope.

The test may be applied to stomach contents, cerebro-spinal and other body fluids under the same conditions as with urine.

Blood in faeces. Rub up a small quantity of the faeces—about the size of a pea—with 2–3 cc. of water in a test-tube, and heat to over 60°, then add 2–4 drops of glacial acetic acid, shake and cool. Take one drop of the watery extract and test as with urine.

In testing faeces for blood it is much better, however, to make and test an ethereal extract as follows.

Rub up a small quantity of faeces with 2–3 cc. of water in a test-tube and heat on a water-bath to over 60° for about 5 minutes, then cool and add 2–4 drops of glacial acetic acid. Shake the mixture and then add about 3 cc. of ether and shake again. Test the separated ethereal extract in the same way as urine.

Much greater sensitivity can be obtained by decanting the ethereal extract and evaporating it on a water-bath at 60°, then treating the residue with one or two drops of distilled water and testing this moistened residue. This latter method can of course be applied to body fluids. In the case of urine it has been found that, by centrifuging the specimen and examining the deposit in this fashion, 1 part of blood can be detected in 3,000,000 parts of urine. Iodides, pus or enzymes do not interfere with the test when performed in this way.

SUMMARY.

1. "Hyperol," a dry stable powder, containing 35 % of hydrogen peroxide, can be used as the oxidising agent in the benzidine test for blood in clinical laboratory practice.

2. By its use the performance of the test is simplicity itself and the cost is almost negligible.

3. The test so performed is thoroughly reliable and extremely sensitive.

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CXXXIII. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

V. PHYSICO-CHEMICAL ASPECTS OF MILK SECRETION.

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From the Hannah Dairy Research Institute, Kirkhill, Ayr.

(*Received June 9th, 1932.*)

The tendency to regard secretion as an osmotic phenomenon has, as yet, found very little expression in the particular case of the secretion of milk. Beyond the fact, reported by Van der Laan [1915] that milk is isotonic with blood, no data on the osmotic relationships of the mammary gland appear to have been established. Taylor and Husband [1922], it is true, have suggested that the flow of milk through the mammary gland is the result of osmosis induced by the elaboration of lactose. Osmosis could, however, only be induced by this means if the lactose were derived from a larger molecule such as the glycoprotein suggested by Hammond [1913]. At the present time there is no evidence for the existence of such a substance in the mammary gland, and it seems more reasonable to assume that a large part of the blood-glucose, shown by us to be absorbed by the gland [Blackwood and Stirling, 1932, 1], is transformed directly into lactose. The synthesis of lactose would therefore involve a decrease and not an increase in osmotic pressure as suggested by Taylor and Husband. This fact demonstrates that definite knowledge of the nature of milk precursors is a pre-requisite for the study of the physico-chemical basis of milk secretion.

In previous papers of this series we have discussed the nature of milk precursors. In the present communication an attempt will be made to consider their absorption and subsequent fate from a physico-chemical standpoint. In such a treatment it is logical to consider first the transference of material from the blood-stream to the gland cells.

The nature of the transudate from the blood-stream to the gland cells.

The first stage in milk secretion must be the passage of a solution of milk precursors from the blood-stream to the secreting cells of the mammary gland. This might be brought about in one of two ways. (1) The intervening cell membrane might be freely permeable to all blood-plasma constituents which

[1] Carnegie Research Scholar.

are in true solution, in which case the transudate would consist of blood-plasma *minus* its colloids. (2) The cell membrane might be endowed with the property of selective absorption and thus be capable of adjusting the composition of the transudate to the requirements of the secreting cells.

The first hypothesis would imply a filtration through the cell membrane analogous to that which has been demonstrated for the glomerulus of the kidney, since the glomerular filtrate has been shown [Richards and Plant, 1917, 1921] to be identical with blood-plasma *minus* its colloids. There are, however, strong reasons for rejecting the hypothesis that a filtration of this type takes place between the blood-plasma and the cells of the mammary gland. This may be concluded from a consideration of the following experimental data.

In Table I A are given the results of analysis of blood from six lactating cows and in Table I B the analysis of milk from six cows (not the same animals).

Table I.

A. *Blood.*

Amounts mg. per 100 cc. blood			Ratios		
Glucose	NH_2-N	NaCl	Glucose	NH_2-N	NaCl
44	6·92	491	6·36	1	70·96
43	6·57	497	6·55	1	75·65
36	7·16	459	5·03	1	64·10
42	7·65	500	5·49	1	65·36
43	8·46	475	5·14	1	56·82
49	7·15	466	6·85	1	65·18

B. *Milk.*

Amounts g. per 100 cc. milk			Ratios		
Lactose	Protein-N	NaCl	Lactose	Protein-N	NaCl
4·87	0·51	0·17	9·55	1	0·33
4·84	0·55	0·17	8·80	1	0·31
5·98	0·38	0·13	15·73	1	0·34
4·88	0·36	0·16	13·55	1	0·44
4·63	0·55	0·15	8·42	1	0·27
4·88	0·45	0·15	10·90	1	0·32

Blood-glucose was determined by Hiller, Linder and Van Slyke's [1925] modification of the Hagedorn-Jensen method—true sugar being found in this way and not total reducing power. Blood-amino-nitrogen was determined by the Van Slyke manometric method [1929] and chloride by the thiocyanate procedure [Whitehorn, 1921].

Lactose was determined in the milk by the Shaffer-Hartmann iodimetric method [1920], nitrogen by the Kjeldahl method, and chloride by thiocyanate titration.

It will be seen that the ratios for blood, amino-nitrogen : sugar : sodium chloride are approximately 1 : 6 : 65, while the ratios for milk are approximately 1 : 11 : 0·35. It is evident therefore that milk cannot be considered

merely as a concentrated (and transformed) transudate of blood-plasma *minus* colloids, and that, in order to arrive at the typical ratios found in milk, it would be necessary to assume that certain of the constituents of the transudate are reabsorbed into the blood-stream. Such a process is considered to take place in the kidney [Cushny, 1926]. In the latter case, however, re-absorption of water and of certain solutes takes place during the passage of the glomerular filtrate along the renal tubules. This implies the existence of two complementary cell structures, one for filtration and one for re-absorption. The histological picture of the mammary gland presented by Martin [1919] shows, however, that the blood capillaries have direct access to the secreting epithelial cells and that no complementary structures akin to the renal glomeruli and tubules are present. The mammary gland is therefore not provided with any apparent means of securing specific re-absorption of part of the transudate.

That this criticism is a serious one may be shown from the following quantitative data. The sugar content of the blood of the lactating cow may be taken as roughly 40 mg. per 100 cc. The experimental animals used by us secrete per 12 hours about 10 litres of milk containing 450 g. of lactose. In order to furnish this amount of lactose there would have to be filtered from the blood in 12 hours $\frac{450{,}000 \times 100}{40 \times 1000} = 1125$ litres of transudate. Thus, on the filtration hypothesis, of this 1125 litres of transudate 10 litres would appear as milk, while the remaining 1115 litres would be re-absorbed into the blood-stream. This hardly appears to be a likely process.

In our view the nature of the transudation is best explained by the second hypothesis, namely, the selective absorption of milk precursors at the membranes separating the secreting cells from the blood supply. In this connection it might be observed that the existence of a renal threshold value for such a blood solute as glucose is explained by preferential absorption of this substance by the cells of the tubules. Further, the substances which are most easily absorbed by the mammary gland (glucose, amino-acids) are precisely those for which renal threshold values exist. We suggest therefore that the membranes separating the cells of the mammary gland from the blood-stream have similar properties to those separating the blood-stream from the renal tubules. Such a similarity would account for the free passage of amino-acids and glucose into the mammary gland and for the comparative impermeability to inorganic salts such as sodium chloride. The postulated preferential absorption would not appear so complex if it could be shown that the amino-acids and sugar were absorbed into the mammary gland in the same ratio as that in which they occurred in the blood. Blackwood [1932] has, in fact, provided positive evidence on this point. Further support for this suggested resemblance of transudation in the mammary gland to tubular resorption in the kidney is to be found in the behaviour of phloridzin. Phloridzin has the property of paralysing the re-absorptive power of the renal tubules for glucose. We should therefore expect that administration of phloridzin to the lactating animal would be followed by diminution in lactose secretion.

This has been noted by Paton and Cathcart [1911] and others.

We suggest therefore that the first stage in milk secretion is the separation from the blood of a transudate which contains the organic milk precursors (glucose and amino-acids) in the proportions in which they occur in the blood, and the inorganic milk constituents, for example sodium chloride, in much smaller relative concentration.

We may mention in this connection our previous finding [Blackwood and Stirling, 1932, 2] that inorganic phosphate is not absorbed by the mammary gland. Since, as has been pointed out above, the supposition that the transudate contains glucose in the same concentration as in blood does not appear likely, it follows that the transudate must be more concentrated with respect to glucose and amino-acids than is blood.

It is appreciated that this view of the transudation from the blood-stream to the mammary gland is attended by the difficulties common to all attempts to account for the passage of fluids across physiological membranes. For example, the theory first suggested by the work of Tamman [1896] and Starling [1899] to account for the filtration from the blood-stream to the glomerulus of the kidney, and later developed by Govaerts [1923] and Schade and Claussen [1924] as a general theory of transudation across cell membranes considers the effective filtration pressure to be the hydrostatic pressure of the blood less the osmotic pressure of the plasma-colloids (oncotic pressure). If the membranes of the mammary gland are specifically permeable towards blood-solutes, then those blood-solutes towards which they are relatively impermeable will exert a high partial osmotic pressure in opposition to transudation. If we accept 150 mm. mercury as the typical arterial pressure and 35 mm. mercury as the typical oncotic pressure of blood, we obtain 115 mm. mercury as the effective filtration pressure. According to our observations, the partial osmotic pressure of sodium chloride in cow's blood is about 1·7 atmosphere or 1300 mm. mercury. It is difficult to see how transudation can be effected against such an oncotic pressure for sodium chloride. For the present, it must suffice to conclude that, in the mechanism of filtration across physiological membranes, considerations exist regarding the nature of which it is not at present justifiable to speculate.

Intracellular osmotic changes in the mammary gland.

Turning now to consider the nature of the osmotic changes produced in the transudate in the gland cells, it is of primary importance to recognise that, no matter what be the osmotic pressure of the transudate from the blood-stream to the gland, the onset of synthetic activity within the secreting cells will be accompanied by a lowering of osmotic pressure. The formation of one molecule of lactose from two molecules of glucose and the elaboration of osmotically inactive colloidal protein from osmotically active amino-acids are reactions involving lowering of osmotic pressure. The formation of insoluble colloidal calcium phosphate would have a similar tendency, but the

direction of the osmotic change involved in fat secretion is more difficult to judge. The precursors of milk-fat are probably of the nature of phosphatides and as these exist in the plasma in the colloidal state they will contribute but slightly to the osmotic pressure of the blood. If they are absorbed into the gland cells in a colloidal state, and if the reactions by which fat is formed take place out of true solution the secretion of fat will involve very little alteration in tonicity within the secreting cells. It is known that the fat content of milk is readily affected by external factors which have no influence on the concentrations of lactose and protein in the milk secreted. The synthesis of these latter constituents involves osmotic changes and their secretion will be controlled by osmotic relationships. The variability of the fat content of milk seems, accordingly, to indicate that fat secretion is unconcerned with the osmotic conditions in the mammary gland. It seems feasible therefore to consider that by far the largest part of any osmotic change taking place during milk secretion is to be accounted for by the synthesis of protein and carbohydrate.

Since milk is isotonic with blood, we have to consider how this lowering of osmotic pressure due to synthesis is related to the osmotic pressure finally attained during secretion—namely the osmotic pressure of milk. It will be apparent that the osmotic pressure attained at the end of synthesis will be a function of the amounts of glucose and amino-acids. It seems possible therefore to obtain data upon which to consider the osmotic relationships of the mammary gland by determining the freezing-point, lactose and protein contents of the milk secreted. In order to obtain such data we have collected and weighed the daily secretion of milk from six animals. The freezing-point of the milk has been determined by the Hortvet cryoscopic method [Hortvet, 1921] together with the amounts of water (by evaporation), lactose [Shaffer and Hartmann, 1920] and protein (Kjeldahl) secreted in 12 hours. These are shown in Table II. With the data available we propose to consider the nature

Table II.

Cow No. ...	1	2	3	4	5	6
Total yield of milk (g.)	8164	8164	9979	9298	10433	10866
Water (P) (g.)	7202	7129	8734	8174	9138	9429
Lactose (a) (g.)	389	387	586	443	471	519
Protein (b) (g.)	260	281	240	211	355	303
Depression of freezing-point (Δ)	0·531°	0·545°	0·544°	0·546°	0·545°	0·543°

of the osmotic changes involved in a hypothetical process the reverse of synthesis—namely the transformation of lactose back into glucose and protein into amino-acids. By this means it is possible to calculate the theoretical composition of the transudate from which the milk is derived.

One gram molecule (342 g.) of lactose dissolved in 1000 g. of water will have an osmotic pressure of 22·4 atmospheres at N.T.P. This is represented by a depression of the freezing-point of 1·86°. If the lactose is now transformed

into glucose there will be an increase in the depression of the freezing-point of approximately 1·86°. The increase for a g. lactose in P g. water will be:

$$1{\cdot}86 \times \frac{1000}{P} \times \frac{a}{342} = \frac{5{\cdot}44\,a}{P}.$$

A similar expression may be developed for the transformation of protein into amino-acids. In column 1 of Table III are given the amounts of the

Table III.

	Yield from 100 g. caseinogen g.	Molecular weight	Yield from 100 g. caseinogen g.-mol.
Glycine	0·4	75·06	0·00533
Alanine	1·8	89·07	0·02020
Valine	7·9	117·10	0·06745
Leucine	9·7	131·11	0·07399
Aspartic acid	4·1	133·07	0·03080
Glutamic acid	21·8	147·08	0·14820
Hydroxyglutamic acid	10·5	163·08	0·06437
Serine	0·5	105·07	0·00476
Proline	8·0	115·08	0·06950
Hydroxyproline	0·2	131·08	0·00153
Phenylalanine	3·9	165·10	0·02417
Tyrosine	6·5	181·10	0·03589
Cystine	0·3	240·26	0·00125
Arginine	5·2	174·15	0·02985
Histidine	2·6	155·10	0·01670
Lysine	7·6	146·13	0·05200
Tryptophan	2·2	204·12	0·00108
Total	93·2	—	0·64707

various amino-acids to be obtained by hydrolysis of 100 g. of caseinogen. The figures have been taken from Mitchell and Hamilton [1929] and while they do not represent a quantitative recovery they may be taken as an approximation. From these have been calculated the g.-molecular yield of each amino-acid (column 2). It will be seen that the hydrolysis of 100 g. of caseinogen will increase the osmotically active concentration of any solution by approximately 0·647 g.-mol. For b g. of caseinogen dissolved in P g. of water the increase in depression of the freezing-point will therefore be

$$1{\cdot}86 \times \frac{1000}{P} \times \frac{0{\cdot}647\,b}{100} = \frac{12{\cdot}02\,b}{P}.$$

This expression may be used to give an approximate idea of the change in osmotic pressure caused by the hydrolysis of milk-protein.

If we have a solution of depression of the freezing-point Δ, and containing P g. of water, a g. of lactose and b g. of protein, the depression of the freezing-point (Δ_1) after hydrolysis of the lactose and protein will be given by the formula

$$\Delta_1 = \Delta + \frac{5{\cdot}44\,a}{P} + \frac{12{\cdot}02\,b}{P}.$$

This value Δ_1 has been calculated for each of the six milks the analyses of which are shown in Table II. The values for Δ_1 are given in column 6 of Table IV. We have now to consider the relationship of these calculated values to osmotic phenomena within the mammary gland during secretion.

Table IV.

Cow No.	P	Q	a	b	Δ	Δ_1	Δ_2	W Cals.
1	7202	17080	389	260	0·531°	1·259°	0·244°	−0·650
2	7129	17190	387	310	0·545°	1·314°	0·225°	−0·688
3	8734	19910	586	349	0·544°	1·240°	0·239°	−0·722
4	8174	17240	443	307	0·546°	1·152°	0·259°	−0·541
5	9138	21670	471	355	0·545°	1·292°	0·230°	−0·844
6	9429	19600	519	303	0·543°	1·129°	0·213°	−0·592

It will be appreciated that, at the end of the hypothetical hydrolysis considered above, the milk constituents are in the form in which they were obtained from the blood. In other words, by a process the reverse of that which must actually take place in the mammary gland, we have regained the transudate stage. The depression of the freezing-point (Δ_1), calculated for each milk, will be that which the transudate from the blood-stream must have in order that the osmotic pressure reached after synthesis has taken place may be that of milk. We may conclude that in order that the secretion at the end of the synthetic stage may be isotonic with blood the transudate must have an osmotic pressure of about three times that of blood. If the value is greater than this, then at the end of synthesis, water will have to be absorbed from the blood to give a product isotonic with blood; if less, then, before the milk can become isotonic with the blood, water will have to be returned to the blood. It has been suggested by Hammond [1913] that an osmotic flow of milk through the mammary gland might be produced by the breakdown of a glycoprotein precursor into lactose and milk-protein. If such a process were to take place it is certain that synthesis of glycoprotein must first take place with consequent lowering of osmotic pressure. A flow of water could result from hydrolysis of a glycoprotein only if the osmotic pressure reached at the end of the synthetic reactions were equal to or greater than that of blood. As has been noted above, this requires the separation of a transudate of osmotic pressure much higher than that of blood. When we remember the many mechanisms utilised by the living organism for the preservation of intracellular osmotic equilibrium, the separation of an intracellular fluid from the blood of higher osmotic pressure than the blood itself seems a very unlikely process.

In view of the isotonicity of blood and milk it appears to us a more profitable hypothesis to consider that at no point in mammary tissue does the osmotic pressure rise above that of blood. On this view the first stage in milk secretion is the charging of the gland cells with a transudate isotonic with blood. Synthesis will then commence and, in order to preserve osmotic equilibrium, water will be returned to the blood-stream. When synthesis is complete, the cells will be filled with milk isotonic with blood. On this view it is impossible to account for the discharge of milk into the alveoli by an osmotic effect. When, however, we remember the difficulties which have beset attempts to explain the continuous flow of other secretions by osmosis, it

seems advisable to ascribe this phenomenon, so far as milk secretion is concerned, to agencies not yet elucidated. It is then possible to test the likelihood of this suggested mechanism of milk secretion in the light of experimental data presented in this paper.

For example it is possible to calculate the amount of transudate required according to the hypothesis for the secretion of a known amount of milk. For this purpose we shall assume that in the gland cells the return of water to the blood takes place after completion of synthesis, whereas in practice the two processes will take place simultaneously. We have previously seen that hypothetical hydrolysis of the protein and lactose in milk gives a fluid of higher osmotic pressure than milk. Since the transudate is considered to be isotonic with blood, the amount of water which must be separated from transudate during milk secretion is identical with that which must be added to the hypothetical fluid to bring the osmotic pressure to the same value as that of the blood.

Let P = g. of water secreted in milk in time t.
Q = g. of water in transudate in time t.
Δ = Depression of freezing-point of the blood.
Δ_1 = Depression of freezing-point of the milk obtained after hydrolysis of protein and lactose.

Then $$\frac{Q}{P}=\frac{\Delta_1}{\Delta},\ Q=\frac{P\Delta_1}{\Delta}.$$

The values of Q for the six milks examined have been calculated and are given in column 2 of Table IV. It will be seen that, on the theory suggested above to describe the various phases of milk secretion, approximately 2/3 of the water obtained by the mammary gland in the form of the transudate will be returned to the blood during synthesis, the remaining third appearing in the milk. While the figures can only be taken as rough approximations, it cannot be said that the theory imposes on the mammary gland the necessity of dealing with excessive amounts of fluid. The theory does of course require that cell membranes shall have a permeability from the blood towards the gland different from the permeability in the opposite direction. This is an assumption which has to be made in describing many biological phenomena.

The energy requirements of the mammary gland.

In a previous paper [Blackwood and Stirling, 1932, 1] we have pointed out the error implicit in the assumption that all the glucose absorbed by the mammary gland is utilised for lactose synthesis. We have suggested that part may be utilised in providing energy and it seems desirable to consider the amount of energy which would be expended by the mammary gland according to the theory of milk secretion suggested in this paper.

Synthetic reactions are considered to be almost thermoneutral, but it is

as well to have some idea of the energy required for the daily synthetic requirements of the mammary gland.

$$\begin{array}{ccc} \text{Glucose} & & \text{Lactose} \\ 2C_6H_{12}O_6 & = & C_{12}H_{22}O_{11}+H_2O \\ 1346{\cdot}4 \text{ Cals.} & & 1351{\cdot}4 \text{ Cals.} \end{array}$$

From the above equation it will be seen that the heat of formation of 1 g.-mol. of lactose from 2 g.-mols. glucose is about 5 Cals. The experimental animals used by us secreted on an average about 1·5 g.-mols. of lactose per 12 hours, as shown in Table II. This will require the expenditure of about 7·5 Cals. which could be provided by the combustion of about 2 g. of glucose. The other synthetic reactions of the mammary gland will also involve the completion of secondary bonds (carbon to oxygen and nitrogen) and it is not to be expected that the energy required for the synthesis of protein and fat will be much in excess of that required for the synthesis of lactose. It is to be concluded therefore that the energy required for synthetic reactions by the mammary gland is so small as to be negligible.

It has been pointed out that the assumption of a transudate isotonic with blood requires the return of water to the blood-stream during synthesis for the preservation of osmotic equilibrium. For convenience of treatment we may consider synthesis to go to completion without separation of water and to be followed by return of water to the blood in amount sufficient to bring the osmotic pressure again to that of the blood. We should thus have a lowering of osmotic pressure due to synthesis and the lowest value obtained would be represented by a new depression of the freezing-point (Δ_2) given by the formula

$$\Delta_2 = \Delta - \frac{5{\cdot}44\,a}{Q} - \frac{12{\cdot}03\,b}{Q},$$

where a, b, Q and Δ have the same meanings as before. Values of Δ_2 have been calculated for the six milks examined and are given in Table IV.

It will be seen that, according to the view expressed in this paper, the secretion of milk involves, in 12 hours, the concentration of a fluid containing Q g. of water and of depression of the freezing-point Δ_2 to milk containing P g. of water and of depression of the freezing-point Δ. Since the process of concentration is considered to take place by passage of water from a solution of low osmotic pressure to one of higher osmotic pressure separated by a semi-permeable membrane it will not entail the expenditure of energy by the mammary gland. This fact is best illustrated by treating the matter formally according to the methods for evaluating osmotic work.

If p_1 = osmotic pressure of the solution to be concentrated,
p_2 = osmotic pressure of the blood,
V_1 = initial volume of solution,
V_2 = final volume of solution,
and W = work done in effecting concentration.

Then $$dW = (p_1 - p_2)\,dv$$

$$W = \int_{v_2}^{v_1} (p_1 - p_2)\,dv = \int_{v_2}^{v_1} p_1 dv - \int_{v_2}^{v_1} p_2\,dv.$$

Since p_2 remains constant throughout the process we may write

$$W = \int_{v_2}^{v_1} nRT dv - p_2 \int_{v_2}^{v_1} dv,$$

$$W = nRT \log_e \frac{V_1}{V_2} - p_2 (V_1 - V_2).$$

Instead of V_1 and V_2 we may write Q and P since these are the amounts of water (in g.) in the transudate and milk respectively. p_2, the osmotic pressure of the blood, is the same as that of milk and at body temperature (37°) is equal to $\frac{22{\cdot}4 \times 310\,\Delta}{1{\cdot}86 \times 273}$ atmospheres $= 13{\cdot}7\,\Delta$ atmospheres. $n = \frac{\Delta P}{1{\cdot}86}$.

$$W = \frac{\Delta PRT}{1{\cdot}86 \times 1000} \log_e \frac{Q}{P} - \frac{13{\cdot}7\,\Delta}{1000}(Q - P) \text{ litre atmospheres}$$

$$= \frac{2{\cdot}303 \times 0{\cdot}082 \times 310}{1860} \Delta P \log_{10} \frac{Q}{P} - \frac{13{\cdot}7\,\Delta}{1000}(Q - P) \text{ litre atmospheres}$$

$$= 0{\cdot}03148\,\Delta P \log \frac{Q}{P} - 0{\cdot}0137\,\Delta\,(Q - P) \text{ litre atmospheres}$$

$$= 0{\cdot}000762\,\Delta P \log \frac{Q}{P} - 0{\cdot}000332\,\Delta\,(Q - P) \text{ Cals.}$$

This expression has been evaluated for each of the milks for which analytical data are available, the values of W so calculated being given in Table IV, column 8. In each case a negative value is obtained. In this fact we have a mathematical expression of the statement made above that the theory of the secretion of milk presented does not require the expenditure of any energy other than that required for synthesis.

It should be noted that when an energy balance is struck according to the equation:

Total energy requirements = energy for synthesis + energy for concentration,

the negative values obtained for the last factor are insufficient to give the total energy factor the negative sign. The conceptions are therefore in harmony with the idea of secretion requiring the expenditure of a small amount of work by the gland cells.

Summary.

The absorption of milk precursors by the mammary gland and the mechanism of milk secretion are considered from the physico-chemical standpoint.

The nature of the transudation from the blood-stream to the mammary gland may best be explained by the hypothesis of selective absorption of milk precursors at the membranes separating the secreting cells from the blood supply.

It is considered that during milk secretion a transudate is separated from the blood having the same osmotic pressure as blood, and that during synthesis water is returned to the blood to maintain the tonicity of the cell fluid. At

the end of synthesis the gland cells will therefore be filled with milk isotonic with blood.

Quantitative aspects of this theory of the mechanism of milk secretion are considered in the light of analytical data for the composition and yield of milk from six cows.

It is shown that this theory of milk secretion does not require the expenditure of any energy by the gland cells other than that necessary for the synthesis of milk constituents.

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CXXXIV. ON THE FUNDAMENTAL NATURE OF PARATHORMONE ACTION.

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In a preliminary paper [McGowan, Cunningham and Auchinachie, 1931], certain tentative conclusions were drawn regarding the intrinsic and essential nature of vitamin D action. A series of complete calcium and phosphorus balance experiments[1] on rabbits has since been carried out with the result that these provisional conclusions have been substantiated in full. The detailed description of these observations will be published in a subsequent paper. The chief deduction drawn at the time was that vitamin D produced its effect by breaking up the lipoids of the tissues and setting free phosphoric acid which was neutralised *in situ* by the calcium of the blood, thus bringing about local deposition of tricalcium phosphate. It should be noted that such precipitation is extra-vascular. A precipitation of calcium salts in various parts of the body following the injection of parathormone has been observed by various investigators [*e.g.* Taylor *et al.* 1931], and it seemed of interest to try to locate the exact site of this deposition. It was thought that this might throw some light on the mode of action of parathormone as also on the vexed question as to whether vitamin D acts primarily and solely through the intermediation of the parathyroid gland.

Because of an acquired familiarity with the histology of their organs, fowls were employed in the investigation: while, on account of their insusceptibility to parathormone [Taylor *et al.* 1931], large doses were administered daily to laying hens, the blood-serum of which has already, as is well known, a high calcium content. It was hoped by these means to obtain a definite result even in such a refractory animal.

The parathormone employed was prepared by Eli Lilly and Co., Indianapolis, U.S.A., according to Collip's formula and contained 20 units per cc. It was injected into the pectoral muscles. The fowls were kept in individual cages. They were fed *ad lib.* on a mash consisting of bran 2 parts, sharps 4 parts, maize 2 parts, crushed oats 1 part and soya bean meal $1\frac{1}{2}$ parts, to $10\frac{1}{2}$ cwts. of which mixture had been added 40 lbs. of a mineral mixture. They received also, *ad lib.*, a grain mixture consisting of equal parts of maize, wheat and oats: oyster shell grit and water were freely available.

[1] The analyses were carried out by Messrs William Godden, William H. Williams and Miss B. W. Simpson.

The calcium content of the blood-serum and the phosphorus content of the whole blood were determined from time to time[1], while, at the death of the fowls, sections were prepared from practically every organ and tissue of the body and treated by von Kossa's silver nitrate method for the detection of tricalcium phosphate.

A brief résumé of the findings in the various experimental fowls will now be given. The hens employed were all 2 year old laying White Leghorns.

Hen 611, brought into the cage from free range on 18. iv. 32: breast-bone quite straight, regular, thin and rigid[2]: no eggs had been laid by 20. iv. 32, when the calcium of the blood-serum was found to be 28·1 mg./100 cc. and the phosphorus of the whole blood 6·04 mg./100 cc.: 50 units parathormone were injected on this date and again next day when the fowl was seen to be rather dull. On 22. iv. 32 it was found to be nearly dead but blood was obtained which contained calcium 20 mg./100 cc. and phosphorus 12·8 mg./100 cc. On *post mortem* examination, the most notable features observed were the presence of small white patches of various sizes in the substance of the breast-muscles (the other muscles were not examined), the smearing of the whole surface of the peritoneal cavity with egg-yolk from a burst ripe ovum, and ovary and oviduct in full laying condition.

The nature of the white spots in the muscles is difficult to establish. In the patches, the muscle fibres were markedly swollen and showed considerable disintegration and necrosis. This subject will be touched on again later. As regards the egg-yolk in the peritoneal cavity, the most feasible explanation of its occurrence is that the egg, having burst from its follicle and having missed the oviduct, in its unprotected state is easily ruptured by the movements of the gizzard and intestine *etc.* There is no evidence to suggest that the bursting is brought about primarily by the parathormone, though it seems to have something to do with the failure to get into the oviduct.

The femur and tibia were very sclerosed and contained a very small amount of a pale marrow (hen originally was slightly anaemic). It may be stated here that in none of the experimental hens employed was there any evidence of osteoporosis or any change, atrophy or hypertrophy, of the parathyroids subsequent to injection[3]. The most important finding, however, was discovered microscopically on examining the sections which had been treated with silver nitrate. Deposits of tricalcium phosphate were found intravascularly in the following situations: capillaries of the lungs, especially at the periphery of the lobules near the chest walls, large veins of the liver, *artérioles à housse* of the spleen, large veins and arteries of the spleen (also in the *vasa vasorum* of these arteries) and the capillaries of the glomeruli of the kidneys (see Plate IV).

The distribution of the condition just described was very irregular in individual organs; and while in this fowl all the organs just mentioned were affected, in others of the series certain of these organs escaped. In every case, however, the lungs were involved. Unfortunately, the intestines of this bird were not examined for calcium deposition. The aorta was quite healthy. All the tricalcium phosphate precipitation found was intravascular with the exception of a trace in the tubules of the kidney and a slight occurrence in phagocytes in the periportal spaces of the liver.

Hen 621, brought into the cage from free range on 18. iv. 32: breast-bone normal: laid an egg on 19. iv. 32; and a shell-less one on 20. iv. 32: on this date the serum-calcium was 22·3

[1] These estimations were carried out by Dr D. W. Auchinachie and Mr A. R. G. Emslie.

[2] The condition of this bone has been regularly employed for some time as an index of the state of the skeletal system generally and has been found to be of very great assistance in this regard.

[3] One has observed fairly frequently cases of laying hens on free range with marked osteoporosis and great hypertrophy and greatly increased activity of the parathyroids together with the production of shell-less eggs. As such fowls were receiving large quantities of calcium in their food together with calcium grit, the condition would seem to be due to a lack of absorption of calcium brought about possibly by too low acidity of the intestinal tract.

mg./100 cc. and blood-phosphorus 3·19 mg./100 cc.: she laid a shell-less egg on 21. iii. 32: 50 units parathormone were given on 20th, 21st, 22nd and 23rd. She looked somewhat dull on the 23rd and was found newly dead, at 9 a.m. on the 24th: blood taken at once from the heart contained 14·6 mg./100 cc. calcium. The *post mortem* condition resembled that of hen 611, especially in regard to the white spots in the muscles, the smearing of the peritoneum with egg-yolk and the condition of the ovary and oviduct. There was no osteoporosis and no change in the parathyroids.

Hen 8, brought into the cage from free range on 22. iv. 32: breast-bone normal: serum-calcium 20 mg./100 cc. and blood-phosphorus 2·38 mg./100 cc. No eggs were laid during the experimental period. 50 units parathormone were given on 22. iv. 32 and again on 23. iv. 32; and 20 units on 24th, 25th and 26th, all at 9.30 a.m.: the hen was very dull on the 26th and was killed by bleeding at 2 p.m. The blood-serum contained 14 mg./100 cc. calcium and the whole blood 4·8 mg./100 cc. phosphorus. Again the findings *post mortem* were much as in the two previous cases but it was noticed that all the muscles of the body showed the white spots previously alluded to. There was no osteoporosis and no change in the parathyroids. Microscopically, the changes in the tissues were as in the previous two but less marked than in hen 611.

Hen 319, brought into the cage from free range on 25. iv. 32: breast-bone normal: blood-serum contained 27·3 mg./100 cc. calcium and whole blood 3·9 mg./100 cc. phosphorus: on 26. iv. 32 20 units parathormone; on 27. iv. 32 laid an egg and was given 20 units: on 28. iv. 32 laid an egg and was given 20 units; on 29. iv. 32 no egg; was given 20 units; on 30. iv. 32, 1, 2 and 3. v. 32 no egg, but 20 units each day; on 3. v. 32 serum-calcium 21·0 mg./100 cc. and blood-phosphorus 4·4 mg./100 cc.; on 4, 5 and 6. v. 32 no eggs laid, but 20 units each day at 9.30 a.m. killed by bleeding at 2.30 on 6. v. 32: serum-calcium 31·2 mg./100 cc. and blood-phosphorus 5·1 mg./100 cc. In this case there were no white spots in the muscles, no smearing of peritoneum with egg-yolk but the ovary was in full laying condition and there was an egg, complete with shell in the shell gland. Microscopically, tricalcium phosphate was found nowhere except in the capillaries of the lung. There was no osteoporosis and no change in the parathyroids.

Hen 566, brought into the cage from free range on 2. v. 32: breast-bone normal: an accident prevented the estimation of the serum-calcium but the blood-phosphorus was 6·2 mg./100 cc.: on 2. v. 32 50 units parathormone; on 3. v. 32 laid an egg and was given 50 units; on 4. v. 32 given 50 units; on 5. v. 32 laid an egg, given 50 units; on 6. v. 32 laid no egg, given 70 units at 9.30 a.m.: at 2 p.m. 6. v. 32 killed by bleeding: serum-calcium was 23·0 mg./100 cc. and blood-phosphorus 6·75 mg./100 cc. *Post mortem*, there were no white spots in the muscles, no egg-yolk on peritoneal surface and ovary and oviduct were in full laying condition; there was no osteoporosis and no change in the parathyroids. Microscopically, tricalcium phosphate was found only in the capillaries of the lungs.

It has been noticed that hens 611 and 621, which showed deposition changes so markedly, had greedily swallowed the calcium grit when put into the cages. It was thought that a resultant hypercalcaemia, produced in this way, might have had something to do with the results obtained and a test was carried out in this regard on hen 321.

Hen 321, brought into the cage from free range at 9.30 on 4. v. 32: breast-bone normal: fed at once by hand, a large quantity of oyster shell grit: blood-serum-calcium 29 mg./100 cc. and blood-phosphorus 5·8 mg./100 cc.: fed oyster shell grit again at 2 p.m.; and at 4.30 given 100 units parathormone into breast muscles: at 9.30 a.m. on 5. v. 32 the hen was very ill: it was killed by bleeding: serum-calcium was 18·8 mg./100 cc. and blood-phosphorus 17 mg./100 cc.: *post mortem* there were no white spots in the muscles but the peritoneal surface was smeared with egg-yolk, while the ovary and oviduct were in full laying condition. There was no osteoporosis and no change in parathyroids. Microscopically, deposition of calcium phosphate was found in the lungs and in a few tubules of the kidney. No definite answer to the question therefore was obtained but it has to be noted that the time between the first injection of the animal and its death was short. The experiment could not be repeated as the supply of parathormone was exhausted.

At this stage it was deduced from the observations just described that the parathormone was, in all probability, producing a condition of supersaturation of the blood with tricalcium phosphate and that this substance was separating out in the quieter parts of the circulation, as for instance in the large veins of the liver, while further it was being sifted out of the blood by the ordinary capillaries, wherever they formed a complex network, as for instance in the lungs, and

the glomeruli of the kidney, and also by the sinusoids of the liver and *artérioles à housse* of the spleen. A consideration of the illustrations seems to indicate that this conception was justified. The hypothesis was however put to the test by the intravenous injection into a fowl of a suspension of Chinese ink in saline solution, made by rubbing the solid ink up with the saline in a mortar and subsequent levigation to get rid of the larger particles.

Hen 544, was brought in from free range and injected intravenously by the wing vein with 35 cc. of such a suspension. During injection the comb became quite livid but later regained its normal red colour. The hen was killed 4 hours after injection, having in the meantime laid an egg: sections were prepared from practically every tissue of the body. Microscopically, carbon particles were found in the capillaries of the lung, the sinusoids of the liver and the *artérioles à housse* of the spleen[1] (see Plate IV). No carbon particles were seen in the large veins and arteries of the liver and spleen or in the glomeruli of the kidneys: on the other hand a considerable number were found in the phagocytic cells of the bone-marrow, the distribution being different in these respects from that of the tricalcium phosphate deposited in parathormone-treated hens.

The mucosa of various parts of the intestine of this hen was examined and carbon particles were found in fair quantity, not however in the capillaries but in the phagocytic cells of the tissue surrounding the capillaries in the villi. In this regard it was unfortunate that the mucosa of the intestines of such reacting hens as 611, 621 and 8 were not examined. The negative results obtained from examination of hens 319 and 566 cannot be regarded as conclusive because there was so little precipitation of tricalcium phosphate anywhere in these cases. The occurrence of the carbon particles in the phagocytes in this situation is very suggestive of a possible method whereby insoluble materials, such as iron refuse, tricalcium phosphate *etc.* may be removed from the body, the mobile phagocytes conveying them through between the epithelial cells on to the surface of the intestine.

The lung in this Chinese ink experiment provided another suggestive appearance, in that there were extravasations of blood into the air spaces (see Plate IV). The most feasible explanation of this was an embolic infarction by the carbon particles. The occurrence may serve to explain the extravasations of blood that have been found in the lungs and intestinal mucosa [Taylor *et al.* 1931] of highly susceptible animals, such as the dog, in parathormone and vitamin D poisoning[2].

The observations made in this investigation do not lend support to the idea that vitamin D produces its action primarily and essentially *via* the parathyroid glands. In considering this point, the relevant occurrences in vitamin D poisoning seem to be somewhat as follows. In the first place, a production of free phosphoric acid, as the primary effect of vitamin D action, appears to occur in the tissues which, in turn, attracts calcium from wherever it can, the intestine or bones, for neutralisation purposes. This need for neutralisation is urgent and calcium may be withdrawn from the blood more quickly than it can be replaced from the above sources. Hence a hypocalcaemia may develop which could serve to stimulate the parathyroid to activity, with the liberation of further quantities of calcium from the bones.

A pure parathyroid action could thus replace the original vitamin D action, if the process got out of hand. It should be noted, however, that, in this suggested mode of action, the parathyroid activity is secondary to the vitamin D activity (which can take place, as has been frequently shown, in

[1] The injection of Chinese ink intravenously provides an excellent method for demonstrating the *artérioles à housse* and for distinguishing them from the Malpighian arterioles.

[2] In the latter, although the main action is supposed to be in the tissues, accumulation of insoluble tricalcium phosphate in the blood-stream, as is to be suggested immediately in this paper, is not excluded. Large amounts of tricalcium phosphate are also frequently found in the intestinal mucosa in vitamin D poisoning.

animals deprived of their parathyroids). Further, parathyroid action differs intrinsically from vitamin D action in that it produces intravascular phenomena alone.

At present the most likely mode of action of parathormone seems to be a stimulation of the resorptive activities of the osteoclasts of the bones [*cf.* Hunter, 1930].

It seems possible that one may have been fortunate in these experiments in the selection of such an insusceptible animal as the fowl; for the smallness of the results produced has permitted of clearer observation and interpretation. A greater accumulation of tricalcium phosphate, in all probability, would have obscured the exact site of its deposition. The insusceptibility of the fowl is, in all probability, to be attributed to the fact that the laying hen tolerates with impunity serum-calcium concentrations as high as 25-30 mg./100 cc.

If hen 321, on account of the short duration of the experiment, be excluded from consideration, it will be seen that hens 611, 621 and 8, which showed tricalcium phosphate deposition and white patches in the muscle, also had their peritoneal surfaces smeared with egg-yolk from a burst ovum. This last phenomenon, on the other hand, was absent from hens 319 and 566 which showed tricalcium phosphate deposition in the lungs only and no white patches in the muscles. The following may be an explanation of these occurrences. It seems possible that the escape of the ripe ovum from the ovary may set in motion a train of events for the completion of the egg, one of which would be the provision in the blood of an extra supply of calcium for the formation of the egg-shell. Where this process is consummated by the ovum travelling down the oviduct, the extra calcium would be got rid of in this manner but the case would be quite other where the ovum was burst in the peritoneal cavity.

Taking into account the irregularity of occurrence and distribution of the calcium deposition as a whole in relation to similar occurrences in the muscles together with the actual histological appearances of the white spots, it seems not unlikely that these may be embolic infarcts due to occlusion of capillaries by deposited tricalcium phosphate. This question however is left open in the meantime.

The calcium and phosphorus contents of the blood at various stages are recorded as found but no interpretation of them is attempted at present. It will be observed, however, that, in general, the blood-serum-calcium subsequent to several injections is lower than before injection.

Summary and conclusion.

The injection of large doses of parathormone intramuscularly into laying hens has produced a deposition of tricalcium phosphate inside the vessels as the main result. This, in all probability, is brought about by the parathormone liberating calcium hydrogen phosphate from the bones by a direct stimulation of the activity of the osteoclasts. This appears to be its only mode of

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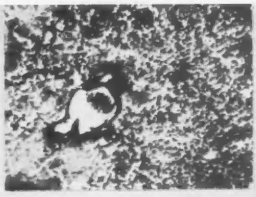

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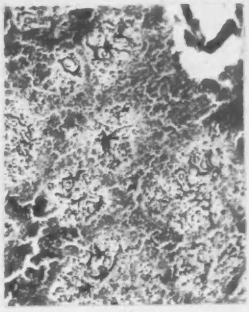

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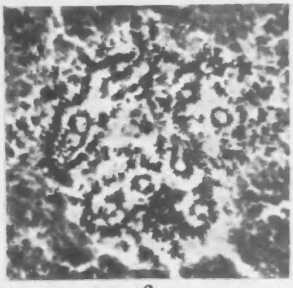

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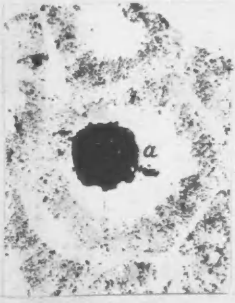

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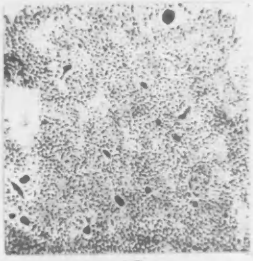

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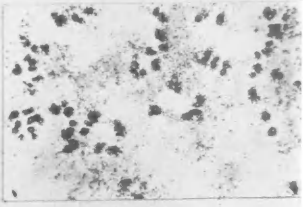

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action. Vitamin D however seems to be able to function in two ways, primarily, by liberating phosphoric acid in the tissues which attracts calcium from the intestine or bones by its acidity, and secondarily, by invoking the aid of the parathyroids through an induced hypocalcaemia.

The parathormone used in these experiments was supplied by Dr Clowes, Director, Eli Lilly Research Laboratories, to whom I wish to express thanks.

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DESCRIPTION OF FIGURES IN PLATE IV

Tricalcium phosphate appears deep black.

Fig. 1. Hen 8. Parathormone injection; lung; silver nitrate; low power; showing very large quantity of tricalcium phosphate in capillaries at periphery of lobules.

Fig. 2. Hen 611. Parathormone injection; liver; silver nitrate; medium power; showing large quantity of tricalcium phosphate in sinusoids and encrusting the walls of the veins.

Fig. 3. High power; vein in Fig. 2 showing the jagged incrustation of tricalcium phosphate. "*a*," blood clot.

Fig. 4. Hen 611. Parathormone injection; kidney; silver nitrate; high power; showing tricalcium phosphate in the capillaries of four glomeruli.

Fig. 5. Hen 611. Parathormone injection; spleen; silver nitrate; medium power; showing several Malpighian corpuscles with their *artérioles à housse* picked out by their tricalcium phosphate content; note absence of tricalcium phosphate from splenic pulp.

Fig. 6. High power of Malpighian corpuscle of Fig. 5; showing tricalcium phosphate in arterioles.

Fig. 7. Hen 621. Parathormone injection; spleen; silver nitrate; high power; showing artery with walls encrusted with tricalcium phosphate (section somewhat oblique).

Fig. 8. Hen 611. Showing vein (seen in Fig. 5) with walls encrusted with tricalcium phosphate; note jagged nature at places.

Fig. 9. Hen 544. Chinese ink injection; lung; unstained; low power; showing carbon particles in capillaries at periphery of lobules; also extravasation of blood into air space (*a*).

Fig. 10. Hen 544. Chinese ink injection; liver; unstained; low power; showing sinusoids filled with carbon particles.

Fig. 11. Hen 544. Chinese ink injection; spleen; unstained; low power showing several Malpighian corpuscles with their *artérioles à housse* picked out by their carbon content: also comparative absence of carbon from the splenic pulp.

(Figs. 9, 10 and 11 are much less highly magnified than the corresponding figures for the parathormone experiments, *viz.* Figs. 1, 2 and 5.)

CXXXV. THE DETERMINATION OF VITAMIN D IN THE LINE TEST BY MEASUREMENT.

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Introduction.

Shipley *et al.* [1921] showed that when cod-liver oil is administered to rats in which a rachitic condition has been induced by a deficient diet, calcium is deposited in the proliferative cartilage of the cartilage-shaft junctions of the long bones, forming a line across the bone at right angles to the longitudinal axis, the amount of the deposit being an indication of the extent to which healing has taken place. On the basis of this observation, McCollum *et al.* [1922] developed a technique for the determination of the ability of any agency to heal rickets, to which they gave the name "line test." In recording the results of tests carried out by this method, McCollum and his associates distinguished four grades of healing from + to + + + +, no healing being represented by a minus sign. Bills and McDonald [1926] later published coloured illustrations of stained bone sections typical of each healing grade. No special quantitative significance was attached to the differences in grade: they were adopted in the first place as a convenience for recording data. However, as data accumulated, Bills was able to establish a relationship between healing and dosage and he published [Bills *et al.*, 1931] a description of an improved technique of the "line" test by which the potency of a substance is estimated as the dose required to produce an average healing of + + in a large number of rats. From this is calculated a cod-liver oil coefficient, from the known dose of average cod-liver oil required to produce + + healing.

Coward [1928] had earlier introduced the use of a standard preparation of vitamin D with which the potencies of other substances could be compared, but this standard had not been adopted in the U.S.A. at the time of Bills's publication.

In the technique of the "line" test described by Dyer [1931] seven degrees of healing (from 0 to 6) are distinguished and the variation between average healing and dosage of the standard preparation has been determined. In the technique for the estimation of vitamin D by radiography described by Bourdillon *et al.* [1931] a similar procedure is followed, but the degree of healing is estimated from X-ray photographs instead of from stained sections, and 13 degrees of healing (0 to 12) are distinguished.

An essential feature of the recently described improved techniques is the estimation of the degree of healing by comparison with an ideal series, but in the method here described, the ideal series is dispensed with, and the degree of healing is expressed as the area of new calcification measured on magnified camera-lucida drawings of the bone sections. This has the advantages that a much finer gradation of healing is distinguished than is possible by the method of eye judgment, and that uncertainty in allotting the healing figure is eliminated. The error in matching the picture given by the test rat is considered by Bourdillon *et al.* to be responsible for one-third of the total probable error of the estimation carried out by their technique. In the technique of Bills *et al.* the difficulty involved in matching the picture against the ideal series is not so important, since only four grades of healing are distinguished.

Experimental.

The breeding stock are fed on a cooked diet consisting mainly of cereal offals with 10 % Trufood-brand skimmed milk powder, 5 % of Gromax and 2 % of red palm oil with 2·5 units of added vitamin D per g. In addition they receive about 10 cc. of milk daily and 10 g. of cabbage 3 times a week. On this diet, the bucks attain an average weight of 200 g. and the does an average weight of 150 g. in 95 days.

When rats are required for test, the mother of a litter is given the stock diet without milk, cabbage or the oil containing vitamin D from 1 week after the birth of the litter. The litters are not reduced in size, and we have not observed any objection to using litters of 10 to 13 rats for the test. The litter is weaned at 23 days, and if the rats are under 40 g. in weight, they are continued on the same diet as the mother had for a week. They are then transferred to the rickets-producing diet (Steenbock 2965), and maintained on it for 3 weeks to a month. An experimental period of 10 days is used. Doses of standard and other oil solutions are administered directly into the mouth from calibrated dropping tubes. Doses of margarine and butter are measured as cut lengths of ribbons obtained from calibrated glass syringes.

The day after the tenth dose has been given, the rats are killed by chloroform vapour, the distal ends of the radii and ulnae are dissected out, and left to harden in formalin overnight. A pair of bones is then sectioned and stained with silver nitrate. The stained sections are covered with water in a small porcelain dish which is placed on a platform attached to the upright of a Giltsch drawing stand. They are viewed through a Zeiss 8× aplanatic magnifier, and the drawings are made with the aid of an Abbé camera lucida. With the distance between the instrument and the drawing paper which has been selected, an overall magnification of 11 diameters is obtained.

The operations of sectioning, staining and drawing, require about 10 minutes per rat.

Measurement of the healing obtained.

When the initial rickets is very severe, the healing produced by a small dose of standard is usually shown by a dense black "line" with well defined edges, as shown in Fig. 1. The area of this "line" can easily be measured by a planimeter.

If the initial rickets is not very severe, or if the dose of vitamin D is large, the newly-calcified area is not completely separated from the shaft of the bone, but it can be distinguished by the pattern of the calcification, usually combining a dense black area with a number of radial lines, as shown in Fig. 2. The actual area of new calcification cannot then be measured so easily,

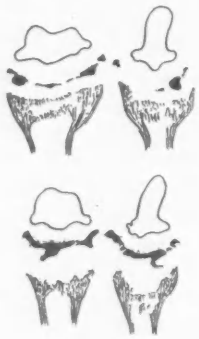

Fig. 1.

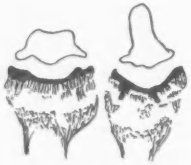

Fig. 2.

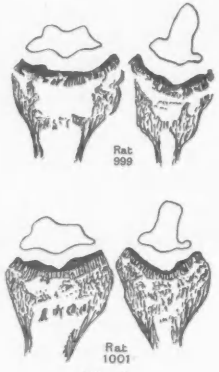

Fig. 3.

Fig. 1. Appearances of healing produced by small doses of vitamin D in rats with severe initial rickets. ×5·5.

Fig. 2. Appearance of healing produced by a moderately large dose of vitamin D. ×5·5.

Fig. 3. Litter-mates which received the same dose of vitamin D ($\frac{1}{2}$ unit). Though the general rachitic condition is more severe in rat 999 than in rat 1001, they show practically the same measured healing. ×5·5.

and it is our practice to measure the total densely calcified area *plus* half the area covered by scattered calcification. In the example given the area of dense calcification (on the original 11× magnified drawing) is 127 mm.^{2}, and the area of scattered calcification is 121 mm.^{2} The figure recorded is

$$127 + \frac{121}{2} = 187 \text{ mm.}^2$$

An illustration of the elimination of uncertainty in estimating healing produced is given in Fig. 3. The pictures reproduced are from litter-mates each receiving 0·5 unit of standard. On the basis of inspection one might place Rat 1001 in a higher grade than Rat 999, since the rachitic condition is less severe; but the measured areas of new calcification are practically equal. Though litter-mates, the two rats showed different degrees of severity of initial rickets.

It sometimes happens that the negative controls show some calcification similar in appearance to the new calcification of healing rickets. This may represent actual healing during the experimental period due to an error in feeding or due to loss of weight by the animals, or it may merely indicate that the initial rickets was not very severe because of a too generous supply of vitamin D in the mother's diet. In the last case the calculation of the assay is not affected (as will be shown below), but in calculating the general average values of the healing produced by doses of the standard preparation (plotted in Fig. 4) a correction has been made by simple subtraction when negative controls have shown such calcification. When the calcification represents healing produced by loss in weight, it is obviously exceptional and does not affect the remainder of the litter.

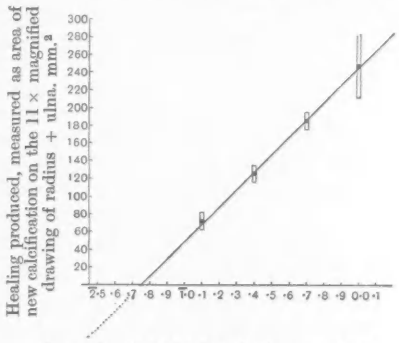

Fig. 4.

The relation between dosage and healing.

The values of the healing produced by different doses of standard in tests over a period of several months have been collected, and the average values are given in Table I. The value for 1 unit is based on the average of 6 rats only. For the three other dosage levels, the standard deviations were in fairly close agreement (29 to 34·5), and the probable errors have been calculated from the general value of the standard deviation determined for the whole series, which equals 33.

Table I.

No. of rats	Dose of standard preparation units	Average healing produced (mm.² on 11 × magnified drawing)	Probable error
44	0·125	72	3·4
58	0·25	125	2·9
43	0·50	185	3·4
6	1·00	247	12·2

In Fig. 4 the average values given in Table I are plotted against $\log_{10}$ dose of standard.

The unshaded area on either side of each point represents three times the probable error of the value. It is seen that the linear relationship fits the figures obtained within the limits of error.

The logarithmic relationship implies that doubling or halving the dose, whatever its value, will increase or reduce the measured healing by a constant

amount. This value has been determined by calculating the average value of the difference in healing between litter-mates receiving doses of vitamin D (whether of known value or unknown) in the ratio 2/1. The value obtained is 59·0 mm.^{2}, and the line given in Fig. 4 has the slope determined by this. The equation to the curve is

$$y = 196 \log x + 245$$

where y is the healing measured in mm.^{2} on the 11× magnified drawing of radius + ulna, and x is the dose of vitamin D in units. In interpreting the results of assays, the value of the constant 245 does not enter into the calculation, since the litters are always divided between the unknown substance and the standard preparation.

Another implication of the logarithmic relationship is the existence of a threshold dose which will just fail to produce any healing. This has frequently been confirmed in tests in which the lower of two doses in the ratio 2/1 fails to produce any healing or produces only a few mm.^{2}, while the higher dose produces healing of the order 60 mm.^{2} The average value of the threshold dose is given by the equation as 0·056 unit.

Interpretation of assays.

Assays may be interpreted by a general method for litters in which the doses are distributed in any way by reducing all the standards to one level and all doses of unknown substance to one level by calculation. The average values are then compared and an assay made for each litter. A weighted average of the separate litter assays is then taken as the final assay. This average must be calculated geometrically (*i.e. via* the logarithms of the values) since variations in doses corresponding to the normally variable healing will not be normally distributed.

Table II. *Assay of a sample of vitamin D concentrate.*

Litter	Rat	Dose	Area of new calcification mm.^{2}	Litter	Rat	Area of new calcification mm.^{2}
146	1103	Negative control	0	148	1118	0
	1110	,,	0		1119	0
	1102	¼ unit standard	92		1115	115
	1112	,,	88		—	—
	1113	,,	103		—	—
	1105	½ unit standard	198		1116	160
	1108	1 unit standard	243		—	—
	1101	$0{\cdot}125 \times 10^{-5}$ g. conc.	119		1117	131
	1107	,,	99		—	—
	1111	,,	127		—	—
	1104	$0{\cdot}25 \times 10^{-5}$ g. conc.	159		1114	147
	1109	,,	167		—	—
	1106	$0{\cdot}5 \times 10^{-5}$ g. conc.	167			

When the litters contain equal numbers of rats on comparable doses of the standard and the unknown, the animals may be considered in pairs and the differences between the individuals of each pair averaged. From this

difference the ratio of the doses may then be calculated by reference to the curve given in Fig. 4 or from the formula

$$(y_1 - y_2) = 196 \log \frac{x_1}{x_2}$$

where $(y_1 - y_2)$ is the difference in healing and x_1/x_2 is the ratio of the doses.

The calculations are illustrated by the assay in Table II.

Method I. (General method for any distribution of doses.)

The average healings produced by $\frac{1}{2}$ unit standard and by $0{\cdot}25 \times 10^{-5}$ g. of concentrate are calculated as follows.

Litter	Rat	Healing produced by $\frac{1}{2}$ unit standard	Rat	Healing produced by $0{\cdot}25 \times 10^{-5}$ g. concentrate
146	1102	92 + 59 = 151	1101	119 + 59 = 178
	1112	88 + 59 = 147	1107	99 + 59 = 158
	1113	103 + 59 = 162	1111	127 + 59 = 186
			1104	= 159
	1105	= 198	1109	= 167
	1108	243 − 59 = 184	1106	167 − 59 = 108
		Average = 168·4		Average = 159·3
148	1115	115 + 59 = 174	1117	131 + 59 = 190
	1116	= 160	1114	= 147
		Average = 167		Average = 168·5

From litter 146 the value of $0{\cdot}25 \times 10^{-5}$ g. of concentrate in terms of standard is given by

$$168{\cdot}4 - 159{\cdot}3 = 196 \log \frac{0{\cdot}5}{x},$$

$$x = 0{\cdot}5/1{\cdot}113$$

$$= 0{\cdot}45 \text{ unit.}$$

From litter 148 the value of $0{\cdot}25 \times 10^{-5}$ g. of concentrate in terms of standard is given by

$$168{\cdot}5 - 167 = 196 \log \frac{x}{0{\cdot}5},$$

$$x = 0{\cdot}5 \times 1{\cdot}017 = 0{\cdot}508 \text{ unit.}$$

The geometrical average of these two values, weighted for the number of rats in each litter (excluding controls) is 0·466 corresponding to

$$0{\cdot}466/(0{\cdot}25 \times 10^{-5}) = 186{,}000 \text{ units per g.}$$

in the original concentrate.

Method II. (Special method when litters are divided into pairs of rats.)

If one of the rats on $0{\cdot}25 \times 10^{-5}$ g. of concentrate had been omitted, the above two litters could have been considered as seven pairs on comparable doses of standard and unknown. Omitting rat 1104, the differences between the pairs are:

$$\begin{aligned} 92 - 119 &= -27 \\ 88 - 99 &= -11 \\ 103 - 127 &= -24 \\ 198 - 167 &= 31 \\ 243 - 167 &= 76 \\ 174 - 190 &= -16 \\ 160 - 147 &= 13 \\ \text{Average} &= 6{\cdot}0 \end{aligned}$$

This average difference corresponds to a dosage ratio of standard to unknown, in any of the pairs, of 1·073, *i.e.* $0{\cdot}5 \times 10^{-5}$ g. concentrate contains $1/1{\cdot}073 = 0{\cdot}932$ unit, or 1 g. contains 186,000 units.

Calculation of errors.

The errors to be expected in practice have been calculated by two of the methods described by Bourdillon *et al.* [1931]. The total number of rats available for consideration, however, is only about 200, and the experiments extend over a period of 7 months. The figures are, therefore, somewhat provisional.

The mean value for the increment obtained on doubling the dose is, as given above, 59·0 mm.^{2} The standard deviation of this value is 30·8 mm.^{2}, *i.e.* the probable error of any result calculated from n pairs of rats is $\frac{2}{3} \cdot \frac{30{\cdot}8}{\sqrt{n}}$.

In many litters several rats have received uniform doses of standard or unknown substance, or have been divided between two levels of dosage. When at least 4 rats have received the same dose, or can be reduced to the same level by calculation, the deviations from the mean for the litter have been calculated. The standard deviation calculated from these figures is 20·6. The standard deviation between two means is thus $20{\cdot}6 \times \sqrt{2} = 29{\cdot}2$, a value agreeing with the standard deviation for pairs of rats receiving doses in known ratio. It is of interest to note that the standard deviation from the mean for animals receiving the same dose is 20·6 if the animals considered are litter-mates, and 33 if the animals are not litter-mates (calculated from values for rats receiving the standard preparation over the whole period). Thus confining the comparisons to litter-mates reduces the probable error of the result by about one-third.

Taking 30 as the value of the standard deviation of the difference between two means, the errors to be expected in an experiment with n pairs of rats have been calculated.

Table III.

No. of pairs of rats	Percentage error corresponding to $\frac{2}{3}\frac{\sigma}{\sqrt{n}}$ "Probable error"		Percentage error corresponding to $\frac{2\sigma}{\sqrt{n}}$ 3 times "probable error"	
1	+27	−22	+102	−51
2	18	16	65	40
3	15	13	50	33
5	11	10	37	27
10	7·7	7·0	25	20
20	5·4	5·1	17	15
30	4·4	4·2	14	12
50	3·4	3·4	11	9·5
100	2·4	2·4	7·3	6·8

The values of percentage error corresponding to $\frac{2\sigma}{\sqrt{n}}$, *i.e.* the error likely to be reached or exceeded once in 22 groups, are plotted in Fig. 5, which shows

also the values calculated similarly by Bourdillon *et al.* [1931] for the estimation of vitamin D by radiography. It is observed that the errors now estimated are about two-thirds those given by Bourdillon *et al.*, which accords with their finding that about one-third of the probable error is due to error in the diagnosis of healing. With this reduction in the probable error, the number of rats required to produce an assay of a given degree of accuracy is approximately halved.

Sources of error.

Errors in measurement. The error in measuring the area of cross section of the new calcification deposit varies according to the type of picture presented. Small areas such as are shown in Fig. 1 can be measured to within a few mm.^{2}, but in measuring larger areas the error is more considerable. It is, however, not likely to be of importance when the healings produced in the animals compared are of approximately the same order. Assays based on a comparison of considerable healing produced by one substance with slight healing produced by the other are necessarily unsatisfactory, and can only be considered as approximate.

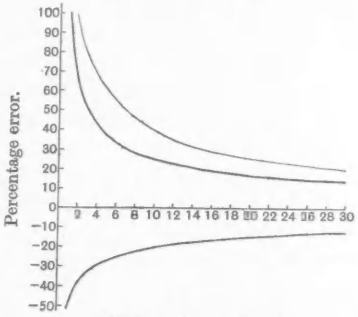

Fig. 5. Relation between error and number of pairs of rats in an assay. The curves show the percentage error corresponding to three times the probable error. The thin line is plotted from the values given for the estimation of vitamin D by radiography [Bourdillon *et al.*, 1931].

Slight errors in measurement may also be caused by the variation in the deposit at different planes of section and by slightly oblique viewing of the bone when making the drawing. These again will only affect to an appreciable extent the measurement of large areas.

Since the relation between healing and dosage is logarithmic, the errors in the assay from these causes are determined by the absolute errors in measurement and not the percentage errors. It therefore appears to be more satisfactory to select doses that produce slight healing ($\frac{1}{8}$ to $\frac{1}{4}$ unit). The variation in response in this neighbourhood does not appear to be greater than at higher levels of dosage.

The error in measurement could be reduced by using a higher magnification in making the drawing and by using both legs of each animal. The extra labour involved, however, does not appear to be justified by the slightly increased accuracy that would be obtained. It might, however, be possible to develop other more accurate methods of measuring the deposit, such as a photometric determination of the average blackness in the area of the line, or a micro-calcium estimation on the complete disc of newly calcified tissue dissected out from the remainder of the bone.

It may be mentioned here that in one routine assay the tibia was used as well as the radius + ulna. The calcium deposits were much less dense than in the radius + ulna, and were less suitable for accurate measurement.

Error due to variation in response. This error is responsible for nearly the whole of the error observed in practice. As is shown above, the error is much reduced by confining comparisons to litter-mates.

Other sources of error. The individual records in a number of vitamin D tests have been examined to determine the effect of the following variables: sex, weight of the animal at the beginning of the experiment, fore-period on the deficient diet, age and growth during the experimental period. None of these appears to exert any considerable influence. Growth during the experimental period appears to be inversely related to healing in many litters, but the effect is not uniform. The results are summarised below.

Influence of sex. The values of the healing produced by the standard preparation in 16 litters were considered. $\frac{1}{8}$-unit figures were converted to $\frac{1}{4}$-unit figures by adding 59, and $\frac{1}{2}$-unit figures were converted by subtracting 59. The healings for bucks and does in each litter were averaged separately and the litter averages were then averaged as follows: bucks 128 mm.^{2}, does 128 mm.^{2}

Influence of weight at the beginning of the experimental period. Considering the same figures, the weights of the rats and the healings were averaged irrespective of sex, and then the deviations from the mean healing for each litter were tabulated separately according to whether the individual exceeded or fell short of the litter average weight. For 30 rats exceeding the average weight, the average of the deviations was -1 mm.^{2} For 29 rats of less than the average weight, the average of the deviations was 0 mm.^{2}

Influence of the length of the fore-period. The values from 22 litters expressed as $\frac{1}{4}$-unit standard were averaged for each litter.

4 litters had a	fore-period of	25/27 days, mean healing	122 mm.^{2}
8 ,,	,,	less than 25 days, mean healing	126 ,,
8 ,,	,,	more than 27 days, mean healing	128 ,,

Influence of age at beginning of experimental period. 23 litters were considered. All were weaned at 23 or 24 days.

9 litters were	less than 29 days from weaning, mean healing	127 mm.^{2}
5 ,,	31 to 33 days from weaning, mean healing	116 ,,
9 ,,	more than 33 days from weaning, mean healing	129 ,,

Influence of growth during the experimental period. The deviations from the mean healings for each litter were tabulated separately according to whether the individual's growth was more or less than the mean for the litter. Of 27 animals showing less than average growth, 16 showed more than average healing and 11 showed less. The average deviations were $-2{\cdot}25$ g. and $+2{\cdot}7$ mm.^{2} Of 27 animals showing more than average growth, 15 showed less than average healing and 12 showed more. The average deviations were $+2{\cdot}0$ g. and $-4{\cdot}9$ mm.^{2}

It is seen that, so far as the figures considered show, none of the variables exerts an appreciable effect, certainly nothing for which an allowance can be made. When a litter is being taken for test, pairs can be selected according to sex and weight, but it is impossible to pair animals according to the growth that they will make during the experimental period. Age and length of fore-period are the same for all the animals of one litter.

Variation in the healing produced by the standard preparation at different periods of the year.

The healings produced by different doses of the standard preparation in routine tests since September 1931, when the method was first applied, have been averaged throughout as $\frac{1}{2}$ unit. The results are summarised in Table IV.

Table IV.

Period covered	No. of litters represented	No. of rats	Mean healing as $\frac{1}{2}$ unit standard
18. ix. 31 to 23. x. 31	6	11	173
26. x. 31 to 18. xi. 31	7	19	186
21. xi. 31 to 21. xii. 31	6	26	187
21. xii. 31 to 6. i. 32	7	15	200
10. ii. 32 to 8. iii. 32	11	28	182
19. iii. 32 to 5. iv. 32	7	21	194
6. iv. 32 to 4. v. 32	10	23	167

During the corresponding period of an earlier year, Bourdillon *et al.* [1931] obtained a variation in healing produced by $\frac{2}{3}$ unit of standard, from about 6 scale divisions in September to 4·7 in February and 8·2 in April, corresponding to a spread of about 100 mm.^{2} in healing measured by the present method. It appears likely that the greater variation obtained is to be explained by a difference in the criterion of healing adopted. The present method does not take into account the general rachitic condition, but only the calcification in the line. As was shown in Fig. 3, two rats may show the same healing while showing general rachitic conditions of different degrees. So long as a line is obtained, the values are not likely to be affected by differences in the degree of severity of rickets developed in the animals before they are taken for test.

Summary.

A method is described for the assessment of the degree of healing in the "line" test for vitamin D by measuring the area of new calcification shown on magnified camera-lucida drawings of the bone sections. In this way a much finer gradation of healing is distinguishable than is possible by the usual method of eye judgment, and uncertainty in estimating the degree of healing is eliminated.

Within the range studied, the healing measured thus is found to be proportional to the logarithm of the dose of vitamin D.

From an examination of the results so far available it appears that the expected probable error in an assay based on 10 pairs of rats is + 7·7 or − 7 %, and the error expected once in 22 tests is + 25 or − 20 %.

The chief source of error is the variable response of litter-mates to a given dose of vitamin. The influence of a number of variables such as sex and weight has been studied, and appears to be negligible.

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CXXXVI. THE ISOLATION FROM BILE OF A PIGMENT HAVING A DIRECT VAN DEN BERGH REACTION.

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The pigments present in the blood-serum in jaundice were first differentiated by van den Bergh, who found that, whereas all icteric sera coupled with diazobenzene-*p*-sulphonic acid (Ehrlich's diazo-reagent) in the presence of alcohol, only certain of these sera would couple in simple aqueous solution. These diazo-reactions van den Bergh termed "indirect" and "direct" respectively, and it is now established beyond doubt that they are to be ascribed to two forms of bile pigment, the so-called indirect- and direct-reacting pigments. Subsequent work has shown that these pigments differ so much in other respects as to justify the conclusion that they are of distinctly different types; the evidence on this point has been summarised by Hunter [1930].

Careful investigation of the conditions under which bilirubin couples with diazo-reagent and comparison of solutions of this pigment in serum with indirect-reaction sera led us to the conclusion that bilirubin is responsible for the indirect reaction [Griffiths and Kaye, 1930, 1, 2], a view first put forward by Collinson and Fowweather [1926] and later by Roberts [1928] and Hunter [1930].

In regard to the nature of direct-reacting pigment, however, there is still much difference of opinion. The views of earlier workers were presented in our previous papers, so that we shall confine our attention to subsequent publications. Hunter [1930] found that if an alkaline solution of bilirubin is first buffered with disodium phosphate, it will couple with diazo-reagent in a distinctly acid medium. On the basis of this observation he suggests that direct-reacting pigment is sodium hydrogen bilirubinate, a view in accordance with that of Collinson and Fowweather [1926], who regarded the pigment as a salt of bilirubin, probably the ammonium salt. More recently, Fowweather [1932] has prepared the mono- and di-sodium salts of bilirubin, and these, when dissolved in serum, were found to give only delayed diazo-reactions. This finding seems definitely to exclude the hypothesis of Hunter [1930], and of Newman [1928], that the pigment is a sodium salt. In this connection it is of interest to recall that van den Bergh and Muller [1916] observed that a neutral solution of bilirubin would not couple with diazo-reagent. Fowweather was unable to demonstrate conclusively that ammonium bilirubinate gives a direct reaction.

Bilirubin is known to exist in more than one physical form, and Fowweather, after careful consideration of all aspects of the matter, has suggested that indirect-reacting pigment is a salt of another modification, more soluble in water, which tends to change into the indirect type. This author describes a pigment giving a direct reaction which is produced by precipitation of a solution of bilirubin in weak alkali, or alkali-phosphate, by a concentrated solution of a similar alkaline salt. This precipitate, when dissolved in serum, was found to give a typical prompt reaction, but on solution in water the product failed to react. The possibility that such a preparation might influence the reaction by virtue of its alkalinity must be considered in view of the fact that the behaviour of bilirubin with diazo-reagent is governed by the p_H of the medium in which the test is performed. Under the ordinary conditions, when equal parts of serum and reagent are mixed, the p_H of the mixture approximates to 2·4; slight variations in technique do not appreciably alter this value. The pigment which couples at this low p_H is of course the direct pigment; this pigment will in fact couple if the p_H lies below about 5·0. Bilirubin does not couple under these conditions, but it can be made to do so if the serum is so buffered, or made alkaline, that the final p_H after addition of the reagent is approximately 5–6. It seems therefore that the mere observation that coupling occurs in an acid medium is insufficient to exclude bilirubin unless the reaction of the medium is shown to be on the acid side of p_H 5·0. In fact, as Fowweather rightly points out, the observation that by addition of sufficient alkali an indirect-reacting serum can be made direct is valueless if, by such treatment, the serum is rendered more alkaline than direct-reacting serum. There can be no doubt that many products described in the literature as direct-reacting were, in point of fact, bilirubin giving a direct reaction because of the alkalinity of the medium in which it was dissolved. We found that when the p_H of serum containing bilirubin was raised to 10 by addition of NaOH, prompt coupling occurred [Griffiths and Kaye, 1930, 1], but for the reason already given such serum is not identical with direct-reacting serum. No artificially prepared serum showing a direct reaction can be considered as identical with the direct-reacting serum of jaundice unless it is shown to couple with diazo-reagent at $p_H < 2$.

Attention must, however, be called to an experiment carried out by Fowweather in which an ammoniacal solution of bilirubin was precipitated with saturated ammonium carbonate solution and the precipitate washed with a saturated solution of sodium chloride; when dissolved in serum the precipitate gave a prompt reaction. In this case most of the alkali must have been removed during the process of washing and further investigation of this interesting observation is clearly desirable.

On the whole, most workers have taken the view that direct-reacting pigment is either bilirubin which has undergone some intramolecular rearrangement, or that it is a salt or other derivative of bilirubin; the evidence which has been put forward in support of these conceptions is not, however, at all

convincing. Curiously enough, practically all the work which has been carried out on the direct-reacting pigment has been synthetic in character, although van den Bergh originally obtained the direct reaction with bile, in which, presumably, the pigment is present in considerable amount. Possibly the difficulties arising from the extreme instability of the pigment have blocked the analytical approach to the problem of its identity. Our knowledge of the physical properties of the pigments, both direct and indirect, and of icteric sera generally, has been much enriched by the many investigations, but it cannot be said that they have brought us anywhere near a solution of the chemical nature of direct-reacting pigment.

In our earlier work [Griffiths and Kaye, 1930, 1] we found that the direct-reacting pigment is present in dried gall-bladder bile after extraction of the bilirubin with chloroform, *i.e.* it is present in that fraction of the bile which is not soluble in chloroform. The preparations with which we were then working were crude, being contaminated by bile salts, *etc.*, but we were able to show that solutions of this material in serum were identical with the direct-reacting serum of jaundice. As a result of further work we have been successful in the isolation from this crude material of a pigment capable of giving a true direct reaction. Briefly, our procedure has been as follows: the chloroform-insoluble residue of dried bile was dissolved in weakly alkaline solution and precipitated by acetone. The direct-reacting pigment is adsorbed by the precipitate, from which it was obtained by extraction of the dried product with 80 % acetone. Evaporation of the acetone solution left a pigmented residue which gave a powerful colour with diazo-reagent at low p_H. The direct-reacting pigment was found to be precipitated from this acetone-product by addition of acids to its aqueous solution.

Experimental.

Human gall-bladder bile, obtained at autopsy, was used. Bile from this source was found to be very variable in its response to diazo-reagent; only those specimens which showed a good prompt direct reaction were employed. When necessary the bile was preserved for short periods in a well-stoppered bottle in a refrigerator from which light was excluded. Light has a very deleterious effect upon the direct-reacting pigment, and for this reason at all stages of the preparation the material was as far as possible preserved in a dark place.

Isolation of the pigment-bile salt complex.

The first stage in the extraction of the direct-reacting pigment was the isolation of a complex substance consisting of the pigment associated with the salt of a bile-acid. The gall-bladder bile was dried *in vacuo* over sulphuric acid and finely powdered. The powdered bile was treated with alcoholic HCl (conc. HCl, 1 cc.; alcohol, 100 cc.) until the mixture was acid to moist litmus paper. In our earlier work this mixture was made acid to Congo red, but

experience has shown that this degree of acidity is not only unnecessary, but distinctly harmful; direct-reacting pigment becomes increasingly unstable with diminishing p_H, and acidity strong enough to affect Congo red may often ruin the preparation. By experiment it was found that a p_H of 6·5–5·5, as determined by the glass electrode, was sufficient to ensure complete solubility of the bilirubin in chloroform without adversely affecting the direct-reacting pigment. After again drying *in vacuo* the finely powdered material was extracted with dry ether for 1–2 hours in a Soxhlet apparatus, using a 22 × 80 mm. thimble; in this way a good deal of pigmented fatty material was removed. Finally, to remove bilirubin, the material was extracted with dry chloroform in a modified Soxhlet. In the ordinary apparatus the chamber containing the thimble becomes considerably heated when using chloroform, with destructive effect upon the direct-reacting pigment; we were able to overcome this difficulty by slightly modifying the apparatus (Fig. 1). Extraction was continued until traces only of pigment were being taken up by the chloroform (6–9 hours). The chloroform was removed from the sticky residue *in vacuo*, and the material powdered. If it appeared that some parts of the substance had escaped extraction the process was repeated for a further period of time. The resulting yellowish-brown powder should give a prompt and marked reaction with diazo-reagent; if it fails in this it is useless to proceed and the specimen must be discarded.

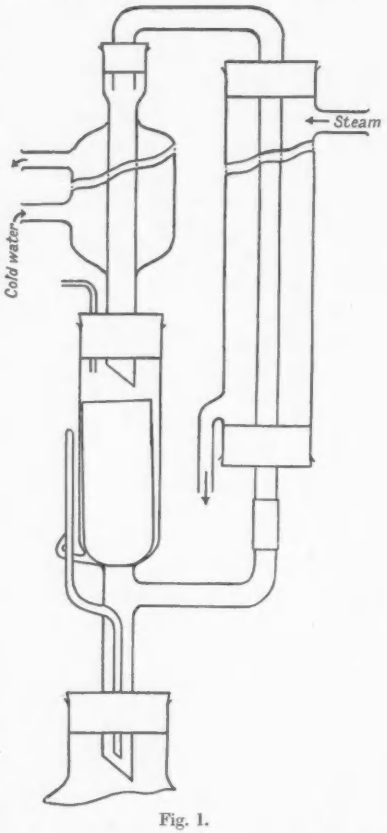

Fig. 1.

The product after extraction with chloroform consisted of the direct-reacting pigment admixed with bile salts, mucin, *etc.*; separation of the pigment from this material proved to be a matter of great difficulty. We found, however, that addition of acetone to an aqueous alkaline solution of the material resulted in the formation of a precipitate, on which, to a very large extent, the direct pigment was adsorbed. We therefore proceeded as follows. The powder was rubbed up with water in a mortar, using about 40 cc. for every 10 g. of material. The dark-brown viscid mixture was made alkaline to

litmus with a few drops of *N* NaOH, transferred to a flask, and precipitated by addition, with shaking, of four times its volume of acetone (distilled from $CaCl_2$). The mixture was shaken vigorously and filtered immediately. The precipitate was taken up in sufficient water to form a cream, and was re-precipitated with another 4 volumes of acetone. The precipitated material was filtered off, washed with acetone, and dried *in vacuo*. The filtrates from these operations, containing mainly bile salts and some pigment, were discarded. The dry acetone-precipitate had an amorphous, sometimes fibrous, appearance, and varied in colour from pale golden-brown to chocolate. It contained N, 7–8 % and P, 1–1·5 %, and was readily soluble in water yielding a solution having a powerful direct diazo-reaction. In the case of strong solutions the colour was masked by a precipitate produced by the reagent, but when this precipitate was filtered off, an intensely purple filtrate resulted, and much azo-pigment could be washed away with water from the residue on the filter-paper. It appeared from this that the substance was split by the reagent, probably because of the acidity of the latter. This was confirmed by the observation that an aqueous solution of the material was precipitated by the reagent acid alone. An equal volume of van den Bergh's solution A was added to a solution of the substance precipitated by acetone, and after being allowed to stand for some minutes, the mixture was filtered. Addition of reagent to the feebly coloured filtrate gave only a very weak reaction, whereas the precipitate reacted strongly with the reagent. The precipitate rapidly lost its reactivity on being left exposed to the air.

The material precipitated by acetone consisted in the main of pigment and mucin. By experiment it was found that the pigment was best extracted from the dry powder by cold 80 % acetone. The material was finely powdered, rubbed up with successive quantities of about 20 cc. of 80 % acetone (80 cc. anhydrous acetone made up to 100 cc. with water) and allowed to stand for 5–10 minutes after each treatment, when the supernatant fluid was decanted on to a filter. After about 10 washings in this manner very little pigment was taken up by the acetone. This operation was carried out by artificial light. Without delay, the acetone was removed from the dark reddish-brown filtrate under reduced pressure in an atmosphere of coal-gas, the containing flask and its contents being warmed to about 60°. When, on account of frothing, further distillation was impossible, any precipitated fatty material was filtered off and the residue evaporated to dryness over sulphuric acid *in vacuo*. The yield, which was variable, amounted to 1–3 % of the chloroform-extracted bile. This preparation will be referred to as the acetone-product.

The nature of the acetone-product.

The dry residue was a reddish-brown, scaly, amorphous and hygroscopic substance, extremely soluble in water, moderately so in alcohol; when powdered it appeared bright orange in colour. Heated on platinum foil, the substance left a very small infusible alkaline ash. Its aqueous solution was

lemon-yellow or orange-red according to concentration, and gave Hay's test for bile-acids. Solutions in normal serum gave an excellent prompt direct diazo-reaction, and were strictly comparable with direct-reacting serum. The intensity of the diazo-reaction of this product was most striking; weak aqueous solutions, containing as little as 1–2 mg./100 cc., gave an immediate purple colour, at approximately p_H 1·5, on addition of an equal volume of reagent. With more concentrated solutions the diazo-reagent produced a precipitate which obscured the colour of the azo-compound, but filtration resulted in an intensely purple filtrate. If allowed to stand for some time after addition of the reagent and then filtered, a small green residue remained on the filter-paper; this was considered to correspond to the oxidation product already described [Griffiths and Kaye, 1930, 1].

The product exhibited marked instability, especially when in the acid state; the pigment thrown down by acids rapidly became green and lost its power to react with diazo-reagent. Aqueous solutions, which at first were lemon-yellow in colour, rapidly became decolorised on exposure to light and ceased to give a diazo-reaction; preservation in a dark place greatly delayed this change.

Acids generally caused precipitation of strong solutions of the product in water when the p_H was brought below about 3·0, whereby practically all the pigment was removed from solution. The precipitated pigment was soluble in alkalis and contained nitrogen and traces of phosphorus.

As it appeared that the pigment represented a small fraction only of the acetone-product, experiments were made in order to determine the nature of the rest of the material. A fairly strong solution of the acetone-product in water was treated with dilute HCl until precipitation was complete, and after filtering NaOH was added to the filtrate until it was only faintly acid to litmus. The neutralised fluid was evaporated to dryness on a water-bath. A pinkish-white, hygroscopic residue was obtained, readily soluble in water to yield a solution which gave Hay's and Pettenkofer's tests; much of the substance was therefore a bile salt. Microscopically the residue, apart from sodium chloride, appeared to consist only of colourless crystals of roughly hexagonal shape.

The relationship between the pigment and this bile salt was not further investigated at this stage, but it seems probable that the association of these bodies was merely accidental, although it is remarkable that, whereas the greater part of the bile salts is removed during the initial precipitation of the bile-residue with acetone, this fraction constantly escaped elimination. The action of acids, which presumably decompose an alkaline salt of the direct-reacting pigment with resulting precipitation of the pigment, leaving the bile-acid in solution, appeared to be reversible; when by addition of alkali the change was reversed, the pigment re-dissolved, and on evaporation to dryness *in vacuo* no significant alteration in the character of the product could be detected.

Isolation of the direct-reacting pigment.

As the pigment appeared to be readily separated by addition of weak acids to its aqueous solution, this method was adopted for its isolation. Care was necessary, however, in view of the harmful influence of strong acidity, and acetic acid was at first employed. The powdered material was treated with glacial, or with alcoholic, acetic acid, dried *in vacuo*, and the dried material extracted with chloroform in order to remove any traces of bilirubin. The amount of chloroform-soluble material was found to vary considerably in different preparations; some showed practically none, others a fair amount. Although some of this pigment was undoubtedly bilirubin, often much was the direct-reacting pigment, whose solubility in chloroform seemed to vary from preparation to preparation. After extraction with chloroform the products, when dissolved in weak alkali, were highly reactive towards the diazo-reagent, giving a true direct reaction. The material varied in colour from orange-red to dark greenish-brown, probably because of the presence of some oxidation products; total nitrogen (micro-Kjeldahl) estimations on six different samples gave values ranging from 3·7 to 5·5 %, and the preparations could not, therefore, be regarded as of uniform composition.

Better results were obtained by precipitation of an alkaline solution of the acetone-product with dilute mineral acid. The acetone-product readily dissolved in water to give a deep orange-red solution of p_H 7–8. Addition of $N/10$ HCl dropwise resulted in a gradual change of colour to a more greenish hue, and ultimately to definite greenish turbidity or actual precipitation at p_H 3·6–4·0. When precipitation did not occur it was readily induced by addition of a little solid sodium chloride to the solution. The greenish pigment, which appeared reddish-brown by transmitted light, was centrifuged, and the supernatant yellowish-green fluid removed. The precipitate was dissolved in $N/100$ NaOH, and the golden-brown solution treated with just enough $N/10$ HCl to re-precipitate the pigment. It was observed that the pigment separated at the same p_H as in the first case; it is interesting to note that bilirubin is precipitated from its alkaline solutions at p_H 6·8. The precipitate was centrifuged, washed 2 or 3 times with $N/100$ HCl, and dried *in vacuo*.

The product was a greenish amorphous powder, practically insoluble in water, but readily soluble in alkalis and in alcohol, in which it yielded a greenish-yellow solution, which rapidly darkened on standing. Solubility in alcohol was not always complete. In chloroform the solubility was variable, some samples showing practically none, others a fair amount; it was noticed that it was particularly those samples showing incomplete solubility in alcohol which also showed chloroform-solubility, which was thought to be due to bilirubin. Bilirubin being practically insoluble in alcohol, the pigment was treated with this solvent and filtered; a small brown residue on the filter-paper was found to be soluble in chloroform. After evaporation of the alcohol *in vacuo*, the dark-green amorphous residue was practically insoluble in water

and in chloroform, but readily dissolved in alkalis to yield a golden- or reddish-brown solution. When dissolved in a little $N/100$ NaOH, the solution, after dilution with water, gave an immediate and strong reaction with diazo-reagent at p_H as low as 1–2. In this connection we must remark the very unstable nature of the pigment, especially in the presence of free acid; although the majority of the products of the action of acid on the acetone-product gave a good van den Bergh reaction, occasionally a product was obtained which did not appear to react promptly. In such cases it was observed that the reaction mixture was clouded by a fine green precipitate; when this was filtered off, the filtrate was found to be highly coloured by the azo-compound. The green material, the amount of which increased with the age of the specimen when it was exposed to air and light, is an oxidation product, and is not reactive towards the diazo-reagent. The pigment contained no phosphorus, evolved pyrrole when distilled with zinc dust, and rapidly lost its power to give a diazo-reaction on exposure to air and light.

The following analytical data were obtained for preparations from different samples of bile (mol. wt. by Rast's method, N by micro-Kjeldahl): (*a*) M.P. 145–150°, mol. wt. 721, N, 4·6 %; (*b*) M.P. 142–150°, mol. wt. 688, N, 3·9 %; (*c*) M.P. 145–150°, mol. wt. 696, N, 4·5 %; (*d*), (*e*), (*f*) and (*g*) N, 3·4, 4·3, 4·3, 4·7 % respectively. The probable error in the nitrogen determinations is large on account of the small amounts of substance available for analysis. Micro-analysis of samples (*a*) and (*c*) showed: (*a*) C, 60·4; H, 7·71; N, 4·35 %; (*c*) C, 64·0; H, 8·48 %; $C_{32}H_{50}O_{11}N_2$ requires C, 60·2; H, 7·89; N, 4·39 %. Further investigation at this stage was precluded by the small amounts of material available. It is obvious that there are discrepancies in these results greater than the probable experimental error, which is not surprising in view of the instability of the pigment, the small amounts obtained, and the fact that some degree of oxidation is unavoidable.

In the absence of more precise data, which must await the investigation of larger quantities of the product, the present results indicate the general nature of the pigment; we feel that there is ample justification for supposing that we were, in fact, dealing with a chemical entity. The low N content is of interest; the N in the samples analysed averaged 4·3 % whereas bilirubin ($C_{33}H_{36}O_6N_4$) contains 9·6 %; this finding alone shows that we were dealing with something other than a modified form of bilirubin. So far as we are aware, there is one other blood-pigment derivative only which is known definitely to contain two N atoms in the molecule, namely, bilirubinic acid ($C_{17}H_{24}O_3N_2$), a reduction product of bilirubin [Fischer and Röse, 1912]. Bilirubin and related derivatives of haemoglobin which have been sufficiently studied contain four N atoms, probably in the form of four pyrrole residues, but in the case of bilirubinic acid two pyrrole groups only remain attached to one another. It seems not unlikely, therefore, that the direct-reacting pigment is similar in nature to bilirubinic acid.

The pigments present in the blood-serum in jaundice have come to be

named after the type of van den Bergh reaction which they give, *i.e.* direct- and indirect-reacting pigments; these terms are clumsy and do not fit in with the nomenclature of the bile pigments. Harrison [1930] has suggested that the pigments should be designated according to their origin in the body by addition of a prefix to the word bilirubin. Thus the indirect-reacting pigment, which is associated with jaundice of the haemolytic type, takes the name of *haemobilirubin*; direct-reacting pigment, which is formed from bilirubin in the liver and excreted along with the bile, Harrison calls *cholebilirubin*. These names are in every way suitable and should, we feel, be brought into common use.

SUMMARY.

The isolation from gall-bladder bile of a pigment having a direct van den Bergh reaction is described. This pigment, for which the name of cholebilirubin is recommended, is shown to exhibit those properties known to be associated with the direct-reacting pigment of obstructive jaundice. Cholebilirubin is not a modification of bilirubin, but a separate and distinct pigment with certain features in common with the other bile pigments; analysis, which must be regarded as preliminary in nature, is in favour of the formula $C_{32}H_{50}O_{11}N_2$.

I wish to express my gratitude to Prof. Hugh MacLean for his encouragement, and to the Medical Research Council for a grant. The micro-analyses were carried out by Dr Schoeller in Berlin.

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CXXXVII. ABSORPTION SPECTRA OF SUBSTANCES DERIVED FROM VITAMIN A.

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In continuation of earlier work on vitamin A we have investigated the ultra-violet absorption of certain products obtained after the vitamin has been decomposed. At the same time, we have tried to elucidate the mechanism of the antimony trichloride colour test.

As is well known, the blue colour is transient, and for purposes of assay it is necessary to determine the intensity of colour during the first minute, as otherwise the rapid fading tends to vitiate the results. Little is known concerning the secondary reactions which occur, and even the primary process is not fully understood. For the full development of the blue colour, the reagent must be nearly saturated and, it would appear, the $SbCl_3$/vitamin A ratio must be very high (order 20,000 : 1). This fact suggests that the blue substance (or substances) is an addition compound; the hypothesis of more than one coloured addition complex has already been advanced to account for the two apparently independent absorption bands seen in the blue solutions [Gillam and Morton, 1931; Heilbron, Gillam and Morton, 1931]. It is possible that the primary process, resulting in the blue colour, leaves the vitamin A molecule intact, whilst the secondary reactions associated with the fading of the colour bring about decomposition. To test this view, the blue solutions have been poured into large volumes of water so that the antimony is precipitated as oxychloride. The organic matter is then recovered and its properties studied.

The general method adopted is as follows. A known weight of liver oil or concentrate dissolved in chloroform is treated with a saturated solution of antimony trichloride in chloroform and the mixture shaken for one minute to allow the blue colour to develop fully. The solution is then poured into water in a separating funnel and shaken vigorously to complete the decomposition of the antimony trichloride. As it is impossible to separate the chloroform layer owing to the precipitated antimony oxychloride, the latter is brought into solution by careful addition of concentrated hydrochloric acid, when separation into two layers readily occurs. The chloroform extract is washed successively with sodium carbonate and water and dried over sodium sulphate. After removal of solvent, the residual oil (or concentrate) is very similar in appearance to the original material and in all cases gives a blue or

purple colour with antimony trichloride, similar to, but weaker than, that given by the original.

An examination of the ultra-violet absorption spectra of the solutions in chloroform reveals, however, as shown in Fig. 1, in place of the broad vitamin A

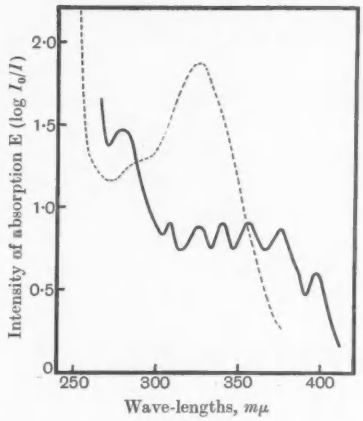

Fig. 1.

– – – – – Cod-liver oil 1 % (1 cm.) alcohol.
——— The same oil "regenerated" 1 % (1 cm.) chloroform.

Table I.

Material	Blue colour with antimony trichloride: $E^{1\%}_{1\,cm.}$	Blue colour with antimony trichloride: Wave-length max. $m\mu$	Blue colour with antimony trichloride: $E^{1\%}_{1\,cm.}$	Blue colour with antimony trichloride: Wave-length max. $m\mu$	Intensity of ultra-violet absorption $E^{1\%}_{1\,cm.}$ $328m\mu$ gross value	Positions of maxima in the ultra-violet absorption spectrum (solutions in chloroform) wave-lengths of maxima in $m\mu$
Cod-liver oil A	1·29	606	1·11	575	1·15	
Do. regenerated	0·96	615	0·77	575	0·72	—, 399, 376, 356, (340), (325), —, (279)
Cod-liver oil B	2·7	603	2·3	573	2·0	
Do. regenerated	1·16	616	1·05	580	1·1	—, 399, 377, 357, 342, 325, 308, (286)
Cod-liver oil C	2·5	604	1·92	572	1·88	
Do. regenerated	1·1	615	1·0	572	0·88	—, 398, 376, 357, 340, 324, 308, 280
Halibut oil	20·4	615	not examined		—	
Do. regenerated	4·6	613	not examined		4·75	—, 402, 378, 358, 340, (324), (309)
Old halibut non-sap.	66·5	616	55·4	583	58	
Do. regenerated	20·7	610	31·9	581	35	(423), 402, 377, 356, 340, (327), —, —
Cod-liver oil non-sap.	213	615	124	580	115	
Do. regenerated	—	no band	92	586	67	425, 400, 379, 357, (340), —, (310), (285)
Halibut concentrate	950	620	order 500	583	370	
Do. regenerated	—	no band	142	590	140	425, 402, 378, 357, (340), —, (309), —
Mammalian liver concentrate	56·5	610	37·4	583	23	
Do. regenerated	4·0	610	6·0	583	6·0	420, 399, 376, 357, 341, 326, 309, —
Cod-liver oil C regenerated in $CHCl_3$	blue colour not studied				—	—, 398, 375, 355, 341, 325, (308), —

band (max. 328 $m\mu$) a series of narrow bands with maxima near 425, 399, 376, 357, 340, 324, 308 and 280 $m\mu$. From the data given in Table I it will be seen that after vitamin A and antimony trichloride in chloroform have reacted to form the highest attainable proportion of blue pigment (about 1 minute), a part of the vitamin A can still be recovered unchanged, although an appreciable proportion has undergone decomposition. The narrow absorption bands shown by the new material make the reaction easy to follow with the spectroscope. The procedure described above has been applied to a variety of fish-liver oils, mammalian liver fats and a wide range of concentrates. The wave-lengths of maximum absorption are always the same, indicating that the decomposition is qualitatively independent of the concentration of vitamin A; the intensities of absorption are always of the same order as the intensity of the vitamin A band in the starting material. Although the 328 $m\mu$ band cannot be observed as such in the "regenerated" material, its presence, masked by the highly selective absorption of the new products, can be inferred with certainty from the general shape of the absorption curve in conjunction with the results obtained by applying the colour test to the recovered materials.

It is interesting to note that the maxima for the new products differ noticeably from those recorded previously for the acids obtained by the hydrolysis of liver oils and concentrates [Gillam, Heilbron *et al.*, 1931] particularly after allowing for solvent displacement of wave-lengths.

	λ max. in $m\mu$									
Regenerated products (in chloroform)	425,	399,	376,	357,	340,	324,	308,	280		
Cod-liver oil acids (in alcohol)		392,	375,	350,	330,	316,	302,	281,	269,	259, 235

The similarity of the resolution into narrow bands may indicate a structural resemblance. That the "regenerated" products are not acidic is, however, shown by the fact that no substances showing narrow absorption bands are extracted on washing with either sodium carbonate or sodium hydroxide. Water is also not essential to the appearance of the narrow bands, since they can be recorded in solutions prepared simply by diluting the blue solutions with much chloroform, making no attempt to eliminate the antimony trichloride.

Other methods for obtaining the substances showing narrow bands.

A. When unsaponifiable extracts from liver oils and fats are dissolved in absolute alcohol containing a little dry hydrogen chloride, the ultra-violet absorption spectrum undergoes a considerable change. For example, a 0·005% solution of a rich halibut-liver oil concentrate, dissolved in absolute alcohol, was found to exhibit the normal vitamin A band, the intensity maximum occurring at $E^{1\%}_{1\,cm.}$ 328 $m\mu$, 1100. The large absorption band was completely free from "fine" structure. When, however, the same concentrate in alcoholic hydrogen chloride was examined, it was found not only that $E^{1\%}_{1\,cm.}$ 328 $m\mu$ had fallen to 575, but the selective absorption had been displaced towards the visible

and a succession of narrow bands was recorded, the maxima being at 419, 392, 369, 350 and 333 $m\mu$. The appearance of this set of narrow absorption bands is shown perfectly clearly with even the richest concentrates, and the conclusion cannot be evaded that these bands are characteristic of a material which is either an isomeride of vitamin A or a product very easily derived from vitamin A.

In order to elucidate the mechanism of the action of hydrogen chloride on vitamin A, we have studied the effect of varying the proportions of vitamin A and acid. Using a 0·006 % solution of rich concentrate containing perhaps 50 % vitamin A, when the concentration of hydrogen chloride is low (order $10^{-3} - 10^{-4}$ N) the narrow bands appear slowly, approaching maximum definition in 1 to 2 days and thereafter gradually disappearing. With a higher concentration of hydrogen chloride ($> N/50$) the narrow bands are fully developed by the time (30 minutes) the spectral absorption can be photographed, but the rate of disappearance appears to be definitely increased. Assuming provisionally that the intensity of the 328 $m\mu$ band for "pure" vitamin A is $E^{1\%}_{1\,cm.} = 1350$, and that the molecular weight is near 300 [Heilbron *et al.*, 1932] it is possible to calculate the relative molecular concentrations of vitamin A and hydrogen chloride. On this basis the range over which our observations extend is [vitamin A]/[HCl] = 1/8000 to 1/0·4. The degree to which the narrow bands can be developed is somewhat variable and there seems to be little doubt that, under the experimental conditions, the substance (or substances) responsible for these bands is somewhat unstable. Many of the differences observed can be readily accounted for by the superposition of the new absorption spectrum on that characteristic of the unchanged vitamin A. The main product, when prepared under favourable conditions, exhibits maxima at 392, 369, 350 and 333 $m\mu$ (see Fig. 2), the 369 $m\mu$ maximum being the most intense. This stage is never attained with very dilute HCl even when in excess over the vitamin, whilst unduly strong acid merely increases the instability of the new product.

The determining factor appears to be the absolute concentration of the HCl rather than the [vitamin A]/[HCl] ratio, since, using the same strength of acid, no difference can be detected in the absorption spectrum whether this ratio is 1/3 or 1/300. On standing, the definition slowly decreases, but this tendency can be arrested, if not entirely avoided, by the addition of sufficient sodium ethoxide solution to neutralise the hydrogen chloride. Similar arrests can be obtained earlier or later in the reaction between vitamin A and hydrogen chloride. It should be remarked, however, that there is no evidence of reversibility, the sodium ethoxide merely tending to stabilise the condition of affairs at the moment it is added.

The best method for obtaining solvent-free material rich in the new absorbing compound is to dissolve 0·5–1·0 g. of a rich concentrate in 100 cc. alcohol and to add enough alcoholic hydrogen chloride to make the solution approximately $N/30$. The mixture is left to stand in the cold for 20 minutes.

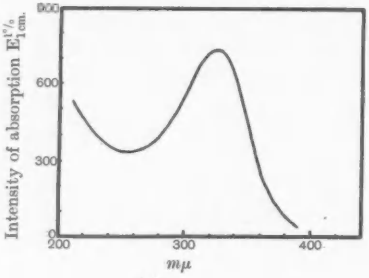

Fig. 2 *a*.

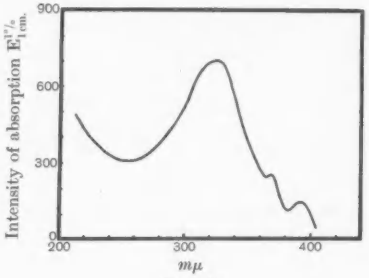

Fig. 2 *b*.

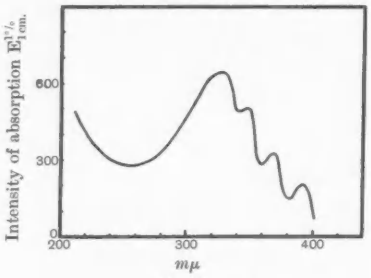

Fig. 2 *c*.

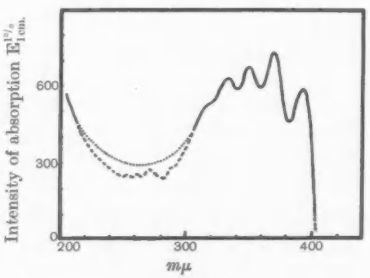

Fig. 2 *d*.

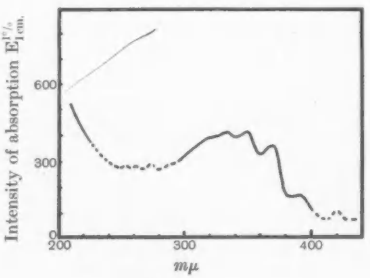

Fig. 2 *e*.

Absorption curves illustrating successive stages in the action of alcoholic hydrogen chloride on vitamin A.

2 *a*. Vitamin A concentrate in abs. alcohol. (Vitamin content about 55 % of that of the richest preparation so far obtained.)

2 *b*. Vitamin A concentrate in alcoholic hydrogen chloride. This curve shows the earliest detectable change.

2 *c*. A later stage in which the alcoholic hydrogen chloride has caused the narrow bands to appear more clearly.

2 *d*. The absorption curve observed at the point at which the narrow bands reach their maximum definition. The curve drawn with a continuous line can be reproduced without difficulty and it should be noticed that the highest absorption occurs at 369 *mμ*. The broken lines indicate that strictly reproducible results in this region of the spectrum are not always obtained.

2 *e*. The absorption curve obtained with vitamin A and alcoholic hydrogen chloride after the mixture (*cf.* 2 *d*) had been left to stand for some days. The poorer definition is worthy of note.

It is then poured into water (400 cc.) and the yellow emulsion extracted several times with small quantities of low-boiling petroleum. After washing with water, and drying over sodium sulphate, the petroleum extract exhibits well developed narrow bands at 392, 369 (highest), 350, and 333 $m\mu$, and ill-defined bands in the regions 316–320, 286–290, 272, 261 and 254 $m\mu$ (these ill-defined maxima are recorded because they have been repeatedly observed, but at the present stage of the enquiry they are much less important than the group 393, 369, 350, 333 $m\mu$). The viscous red-brown oil remaining after removal of solvent exhibits the usual four main bands with great clarity. In an attempt to gain some insight into the mechanism of the reaction leading to this new material, molecular weight determinations, micro-analyses and colour test determinations were carried out and are summarised in Table II.

Table II.

	Vitamin A concentrate	New product
Colour test	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ 3900 580 $m\mu$ 2300	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ 2350 580 $m\mu$ 1400
Micro-analysis	C, 82·8 % H, 10·9 %	C, 82·9, 82·7 % H, 10·4, 10·6 %
Molecular weight (cryoscopic method: solvent benzene)	327	403

The results show that the new product is heterogeneous and that [Heilbron *et al.*, 1932] some polymerisation has probably occurred. The blue value is somewhat higher than would be expected from the ultra-violet absorption.

B. The appearance of narrow bands during the slow high vacuum distillation of rich concentrates is discussed elsewhere. In order to discover how far this effect was due to the prolonged heating, a very rich vitamin A preparation (0·3 g.) was heated in an atmosphere of nitrogen, the temperature being maintained within the limits 120–125°. Small weighed portions were removed from time to time and studied spectroscopically. The results may be summarised as follows. The starting material exhibited the vitamin A band at $E^{1\%}_{1\,cm.}$ 328 $m\mu$, 1100, the absorption curve showing great persistence, as will be realised from the fact that the minimum at 245–250 $m\mu$ occurred at $E^{1\%}_{1\,cm.}$ 300. After 6 hours, the 328 $m\mu$ band had largely disappeared and had been replaced by new absorption in the region 290 $m\mu$. At this wave-length the intensity remained practically constant at $E^{1\%}_{1\,cm.} = 370$ for a further 54 hours' heating, when the experiment was stopped; the definite maximum at 290 $m\mu$ attaining its highest persistence (never very great) after about 10 hours. The minimum was also fairly constant at $E^{1\%}_{1\,cm.}$ 265 $m\mu$, 330. The narrow absorption bands first became detectable after about 10 hours, and reached the highest definition a few hours later, but at best occurred with far less clarity than in the experiments with alcoholic hydrogen chloride or in the fractional slow high vacuum distillation, being little more than fairly sharp inflexions. Substantially, then, the prolonged heating at 120–125° brings about a type of

decomposition associated much more with a substance exhibiting the 290 $m\mu$ maximum than with the narrow bands. If, therefore, the occurrence of these bands in certain distillates is due to heat alone, temperatures above 125° are seemingly essential, and possibly the reaction occurs in the vapour phase. It is interesting to note that while the distillates consisted of pale oils, in our experiments the material grew more and more viscous as the heating progressed, eventually becoming a clear red resin softening at 40–50°. Determinations of the colour test intensities (antimony trichloride) indicated a steady decrease in vitamin A content, but even after 60 hours a perceptible blue colour was given although the intensity had fallen to about 1/40 of the initial value.

Oxidation of vitamin A.

There is general agreement in the literature of the subject that oxygen should be excluded as completely as possible in order to minimise loss during the processes of saponification and extraction of the vitamin from liver oils. During recent months we have repeatedly observed, however, that rich concentrates exhibit a somewhat unexpected resistance to oxidation under certain conditions, and Dann [1931; 1932] has already shown that oxidation of vitamin A concentrates by aeration at the boiling-point in alcohol or alcoholic potash is not achieved in 24 hours. Our own experiments have been carried out on rich halibut-liver oil concentrates dissolved in alcohol. A moderately brisk stream of oxygen from a cylinder was passed through the solution heated under reflux and samples were withdrawn periodically, the vitamin A potency being determined in terms of the intensity of the ultra-violet absorption maximum at 328 $m\mu$. For 8–10 hours the absorption spectrum remained quite constant within experimental error (here $\pm$ 5 %), but after this period the intensity of absorption at 328 $m\mu$ slowly decreased and fine absorption bands with maxima near 392, 369 and 350 $m\mu$ began to appear, not, however, with any great intensity. During the experiment there is a very decided increase in the intensity of absorption on the ultra-violet side of 328 $m\mu$ and this is reflected in a large decrease in persistence. The simplest interpretation of the results is that after a prolonged period of induction slow oxidation occurs coinciding with two changes in spectral absorption, one the appearance of rather unselective absorption in the middle ultra-violet and the other the appearance of highly selective absorption on the long wave side of 328 $m\mu$. The experiments confirm Dann's observation of the high resistance to oxidation of alcoholic solutions of vitamin A, and indicate that the first oxidation product probably exhibits a broad unresolved band near 290 $m\mu$.

As would be expected, ozone is much more effective than oxygen and the vitamin is readily destroyed. In our experiments the object was to ascertain whether any new absorption bands could be recorded after the vitamin had been exposed for a few seconds to small quantities of ozonised oxygen. The reaction proved very difficult to control but certain qualitative observations are of interest. In the first place, exposure of dilute alcoholic solutions of

vitamin A to ozonised oxygen invariably results in a substantial decrease in the intensity of absorption at 328 $m\mu$, but the intensity in the region 250 to 300 $m\mu$ does not fall nearly so rapidly, so that the reaction products must absorb more strongly in the middle ultra-violet than in the region 300–400 $m\mu$. In a number of cases, the exposure to ozonised oxygen was of very short duration, and it seems that the first spectroscopically detectable oxidation (ozonisation) product is characterised by a broad absorption band of small persistence, free from "fine" structure, and exhibiting a maximum at 290 $m\mu$ approx. The intensity of this absorption band is of the same order as that of vitamin A at 328 $m\mu$. In other experiments a maximum at 272 $m\mu$ made its appearance. In all experiments in which a solution of rich concentrate (0·008 %, $E^{1\%}_{1cm.}$ 328 $m\mu$, 1100) was exposed to a fairly slow stream of ozonised oxygen for more than 1 minute at room temperature the resulting solution showed no appreciable absorption over the range 200–500 $m\mu$. In no circumstances was it possible to observe narrow, sharply defined absorption bands as a result of the action of ozone on vitamin A concentrates.

A complete interpretation of the foregoing results is impossible at this stage, but it may perhaps be useful to point out a distinctly interesting analogy emerging from this work and that of Kuhn [1931] on unsaturated compounds [*cf.* also Euler *et al.*, 1932]. Crotonic acid has a broad band with a maximum at 204 $m\mu$, sorbic acid has a similar band with its maximum displaced to 250 $m\mu$, whilst the octatriene and decatetraene acids exhibit bands at 294 $m\mu$ and 329 $m\mu$ respectively (private communication from Prof. Kuhn). Similarly Euler *et al.* [1932] record continuous end absorption on the ultra-violet side of 250 $m\mu$ for sorbic alcohol ($CH_3(CH = CH)_2CH_2OH$), a well defined maximum near 270 $m\mu$ ($\log \epsilon$ 4·75) for octatrienol ($CH_3(CH = CH)_3CH_2OH$) and a maximum near 310 $m\mu$ ($\log \epsilon$ 4·7) for decatetraenol ($CH_3(CH = CH)_4CH_2OH$). Now, it seems significant that the first oxidation product of vitamin A exhibits a maximum near 290 $m\mu$ and the second product a maximum near 272 $m\mu$. By analogy with the data quoted above it is likely that the selective absorption of vitamin A is due largely to a straight chain containing four conjugated double bonds and that the displacement from 310 (decatetraenol) to 328 $m\mu$

Table III.

Material	λ max. ($m\mu$)	*log ϵ max.
Crotonic acid	204	4·08
Sorbic acid	250	4·40
Octatriene acid	294	4·56
Decatetraene acid	329	4·69
Sorbic alcohol	< 240	order 5
Octatrienol	270	4·75
Decatetraenol	310	4·7
Vitamin A	328	4·5 *circa* (calculated on a molecular weight near 300)
1st oxidation product	290	order 4·5
2nd oxidation product	272	—

* ϵ is molecular extinction coefficient.

(vitamin A) is due to the joint action of the fifth double bond and the "loading" effect of the ionone ring. Probably, the first action of ozone is to attack one of the double bonds in the side chain, and then selectively to attack a second. It is interesting that only two intermediate stages in the oxidation can be detected spectroscopically. This is not surprising since sorbic acid has its maximum well in the ultra-violet and sorbic alcohol probably in the more extreme ultra-violet.

Table III illustrates the main points referred to above.

Discussion and summary.

The recovery of vitamin A from the blue solution obtained with antimony trichloride by pouring the mixture into much water has been attempted. Partial decomposition of the vitamin occurs, accompanied always by the appearance of narrow absorption bands with maxima at 399, 376, 357 and 340 $m\mu$ (chloroform), and sometimes by additional, less definite maxima, *e.g.* at 425, 324, 308 and 280 $m\mu$. The wave-lengths at the maxima are constant, irrespective of whether either rich or poor oils, or concentrates are used, but the intensities vary roughly with the potency of the material. Recovery of vitamin A, although never quantitative, supports the view that the initial reaction is the formation of a vitamin A-antimony trichloride loose addition product, but secondary (condensation?) processes cannot be excluded, and probably account for the narrow bands. This view is supported by the observation that the similar maxima 392, 369, 350, 333 $m\mu$ (alcohol) can be obtained with greatly enhanced definition by the action of alcoholic hydrogen chloride on rich concentrates, the intensity, persistence, rate of development and permanence depending on the concentration of hydrogen chloride. When the narrow bands are best shown, the 369 $m\mu$ band is the most intense but even under optimum conditions (15°, *N*/30 HCl, 20 minutes) the reaction is incomplete and the product unstable in acid. Neutralisation of the acid with sodium ethoxide arrests the decomposition of the material, but does not effect reversal to vitamin A.

Similar narrow bands, together with a broad continuous band with its maximum at 290 $m\mu$ are shown in certain fractions obtained in slow high vacuum distillation of rich concentrates heated to about 180°. By prolonged heating at 120–125° in an atmosphere of nitrogen the development of narrow bands could not be induced to more than a minute extent, but the vitamin nevertheless decomposed fairly readily, the main product exhibiting a maximum at 290 $m\mu$. At the boiling-point in alcohol, vitamin A is unexpectedly stable to prolonged exposure to a stream of oxygen. After some hours, the 328 $m\mu$ maximum decreases in intensity, the absorption in the region 260–300 $m\mu$ slowly increases and the narrow absorption bands in the near ultra-violet appear, but with comparatively low persistence. Ozonised oxygen rapidly destroys vitamin A with disappearance of selective absorption but compounds with maxima at 290 $m\mu$ and 272 $m\mu$ can be detected in the very early stages.

The data are consistent with the vitamin A molecule containing a side chain with at least four double bonds, which can be attacked in turn by ozone to give products with broad bands.

The hypothesis of ring closure to give hydronaphthalene derivatives as an explanation of the narrow bands will be discussed in a later communication.

We desire to express our thanks to the Department of Scientific and Industrial Research for a grant enabling Mr R. H. Creed to render valuable assistance in the laboratory work..

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CXXXVIII. THE ISOMERISATION OF CAROTENE BY MEANS OF ANTIMONY TRICHLORIDE.

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The stable blue solution (λ max. 583–590 $m\mu$) obtained by the action of antimony trichloride on carotene in chloroform has been poured into water and the organic matter recovered free from antimony. The product is different from, but isomeric with, carotene (compare "regeneration" experiments on vitamin A [Edisbury *et al.*, 1932].

The carotene used (M.P. 180°) was a commercial sample recrystallised from pyridine. Purified in this way carotene exhibits maxima in the spectral absorption curve (solvent, chloroform) at 495, 462, 436, 348 and 280 $m\mu$. The absorption attains its highest intensity at 462 $m\mu$, at which point the molecular extinction coefficient is 102,000 ($E^{1\%}_{1\,\text{cm.}}$ 1900), in agreement with the results obtained previously for the purest carotene [*cf.* Dulière, Morton and Drummond, 1929]. The intensity of absorption at the maximum (585 $m\mu$) in the colour test with antimony trichloride can be expressed as $E^{1\%}_{1\,\text{cm.}}$ 585 $m\mu = 420$.

The method adopted in the "regeneration" experiments was as follows. Carotene (1 g.) was dissolved in chloroform (100 cc.) and treated with a saturated solution of antimony trichloride in chloroform (140 cc.). The mixture was allowed to stand for two minutes and was then poured into water, whereupon antimony oxychloride was precipitated and the original colour of the carotene returned. The oxychloride was dissolved in the minimum quantity of concentrated hydrochloric acid and the mixture left to stand, when the chloroform layer gradually separated. The aqueous portion was shaken with chloroform to recover every trace of extractable material, and the united chloroform extracts were repeatedly washed with water till free from all traces of antimony, and finally dried over sodium sulphate. After removal of solvent by evaporation in a current of nitrogen, a red crystalline residue similar to carotene in appearance was obtained.

This material was then boiled with pentane (200 cc.) and the insoluble residue (fraction A) removed by filtration. The filtrate was concentrated to half-bulk when a further crop of crystals, M.P. 170°, was deposited (fraction B), while complete evaporation of the solvent gave a crystalline residue, M.P. 140° (unsharp) (fraction C). Recrystallisation of A from pyridine raised the M.P. to 185° which was not altered by further purification.

All three fractions of the regenerated product when dissolved in chloroform gave solutions slightly redder in colour than those obtained with carotene at the same dilution. The absorption spectrum in the visible region was characterised in each case by a group of maxima similar to that shown by carotene, but displaced in the direction of the longer wave-lengths. The absorption in the ultra-violet was totally different from that of carotene (see Fig. 1).

Material	M.P.	Wave-length maxima in $m\mu$						
Gross "regenerated"	—	509	482	454	425	389	367	317
Fraction A	185°	512	483	453	(426)	389	—	316
Fraction B	170°	511	480	450	423	389	367	316·5
Fraction C	140°	510	480	450	(423)	—	—	317
Carotene	180°	497	463	436	—	—	350	280

It will thus be seen that the new material is unmistakeably different from carotene in its optical properties. It exhibits a larger number of narrow absorption bands, and in the visible, where the absorption curve is qualitatively similar to that of carotene, the wave-length displacement is very definite. All three fractions exhibit the same maxima and cannot be differentiated qualitatively.

The regenerated product gives a blue solution with the antimony trichloride reagent exhibiting a maximum at 583–587 $m\mu$, practically identical with that of ordinary carotene. The intensity, however, with the sample of highest M.P. did not reach that obtained with the purest carotene recrystallised from pyridine.

Material	Colour test $E^{1\%}_{1\,cm.}$ 585 $m\mu$	Visible absorption $E^{1\%}_{1\,cm.}$ 462(480) $m\mu$	Ultra-violet absorption $E^{1\%}_{1\,cm.}$ 317 $m\mu$
Carotene	420	1900	—
Regenerated material:			
Fraction A	370	1800	430
Fraction B	365	1800	435
Fraction C	340	1370	330

Without more elaborate purification on a larger scale it cannot be concluded that the intensities of the absorption bands shown by the regenerated product are much different from those shown by carotene itself.

The three main fractions of the regenerated pigment were examined for growth-promoting activity by tests on young rats, the growth of which had been inhibited by feeding on a diet devoid of vitamin A. The basal diet was compounded of

Purified caseinogen ...	20
Rice starch	80
Salt mixture	5
Dried yeast	5

Vitamin D was supplied by the administration of 0·001 mg. irradiated ergosterol twice a week. This, as well as the recovered pigment, was given in the form of a solution in ethyl laurate. The solutions were prepared twice a week and stored in the dark at low temperature. Only one preparation showed any sign of growth-promoting power, namely, that with the highest melting-point (185°). When administered in amounts which in the case of carotene bring about a good resumption of growth, there was an indifferent response (see Fig. 2). The results of tests with larger doses suggested that the material was mainly composed of an inactive substance but that a small amount of an active component, possibly unchanged carotene, was also present.

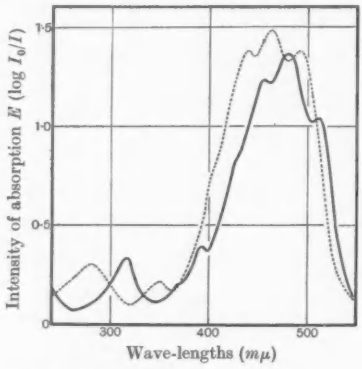

Fig. 1. Spectral absorption curves:
---- Carotene (recryst. pyridine). Calculated to 0·00078 % (1 cm.) $CHCl_3$.
—— Regenerated product. Calculated to 0·00075 % (1 cm.) $CHCl_3$.

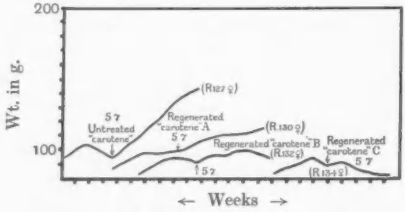

Fig. 2. Biological tests on carotene before and after treatment with antimony trichloride.

The preparations with lower melting-points were devoid of growth-promoting power.

Comparison of the regenerated product with the different varieties of "carotene" studied by Kuhn and Brockmann [1931], and Kuhn and Lederer [1931; 1932] reveals that it is probably identical with *iso*carotene, prepared by the action of either metallic mercury or sodium thiosulphate upon carotene tetraiodide.

	M.P.	Absorption maxima in CS_2, $m\mu$				Growth-promoting activity
α-Carotene	174–175°	511	478	448	—	good
β-Carotene	180–181°	520	485	454	—	good
*iso*Carotene	185–186°	543	503	472	—	nil
Regenerated product	185°	540	505	478	445	nil

SUMMARY.

1. When the blue solution obtained by mixing antimony trichloride and carotene in chloroform is poured into water, the original colour of the carotene reappears in the chloroform layer from which red crystals can be isolated.

2. The absorption spectrum of the recovered product has been determined in chloroform and carbon disulphide. Both in the visible and ultra-violet regions it is quite different from that of carotene.

3. The properties of the substance agree with those of *iso*carotene.

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CXXXIX. CHARACTERISTICS OF HIGHLY ACTIVE VITAMIN A PREPARATIONS.

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In a series of papers emanating from these laboratories during the past five years unequivocal spectroscopic data for vitamin A have been established, and the ground has been cleared for a final comprehensive attack on the problem of isolating the vitamin and establishing its constitution. The present communication embodies the results of work carried out during the last 18 months, and leads us to the view that a definite solution of the problem is now in sight, a view supported by the recent highly important and illuminating publications of Karrer, Morf and Schöpp [1931, 1, 2], to which further reference will be made later.

Vitamin A, both in liver oils and concentrates, exhibits a broad continuous absorption band in the ultra-violet, extending from 260 to 380 $m\mu$, and exhibiting a maximum near 328 $m\mu$. The intensity of this band can easily be measured and used as a definite quantitative test for the potencies of oils and concentrates. Vitamin A also shares with a variety of polyene substances the power of giving a blue solution with a saturated solution of anhydrous antimony trichloride in chloroform. This colour test gains in specificity when the spectral absorption of the blue solution can be accurately described. As a matter of convenience attention may be concentrated on the points of maximum absorption described in terms of intensity and spectral location[1]. Thus the blue solution obtained with vitamin A exhibits two maxima at 617 and 583 $m\mu$ respectively, the former being generally much more intense than the latter, which it tends to mask. In unsaponified oils the maxima are displaced to 606 and 573 $m\mu$ respectively, and here the longer wave-length absorption

[1] The notation used in this paper is necessarily a little different from that employed in spectroscopic studies on pure substances. The usual method is to state the maximum molecular extinction coefficient ϵ, defined by $E = \log I_0/I = \epsilon cd$. ($I_0$ is intensity of incident light, I is intensity of emergent light, E is extinction, c = molar concentration, d = thickness in cm.) In this work $E^{1\%}_{1\,\text{cm.}}$ refers to $\log I_0/I$ at a wave-length maximum, using a solution containing 1 % of vitamin-containing substance and a 1 cm. cell.

suffers considerable but variable diminution in intensity as a result of the inhibiting action of certain normal constituents of fish-liver oils.

In a number of oils and concentrates the presence of additional chromogenic substances manifests itself in the colour test by giving rise to other maxima, among which one at 693 $m\mu$ occurs with considerable regularity [Morton, Heilbron and Thompson, 1931]. With such criteria of vitamin A available it seemed obvious that, provided a satisfactory method of isolation of the vitamin could be achieved, pure preparations from the livers of various species of both fishes and of mammals should all exhibit the maxima at 328, 617 and 583 $m\mu$, each at its own limiting maximum intensity, if all three were indeed properties of vitamin A itself. On the other hand, if any one of the three maxima appeared with relatively diminished intensity, the interpretation would have to be reconsidered. It by no means necessarily follows, however, even if constant values are reached, that vitamin A has been obtained as a homogeneous substance, although one condition of purity is apparently satisfied. It is this stage which we have now reached in our investigations; concentrates of equal potency derived from different sources have been obtained and have proved incapable of being increased in potency by any process calculated to result in a separation of the constituents of a mixture.

Table I.

Material	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	$E^{1\%}_{1\,cm.}$ 580 $m\mu$
Average cod-liver oil	0·78*	0·92	0·70
Typical halibut-liver oils (*a*) winter catches	4·25	7·5	6·0
" " (*b*) winter catches	3·5	6·2	4·8
" " (*c*) winter catches	15·6	32·0	16·7
Total non-sap. (9 %) from (*c*)	380	720	460
Rich halibut oil (*d*)	35	70	47
Non-sap. of (*d*) (approx. 2 %) after freezing at −50°	1150	3300	1800
Rich halibut oil (*e*)	70	148	85
Total non-sap. (8 %) of (*e*)	550	1150	600
(*e*) deprived of sterol at −10° (4·7 %)	1060	2210	1110
(*e*) after treatment at −50°	1150	3020	1450

* Equivalent to 9 Carr-Price units on oil and 17·2 on non-sap.

Earlier work had convinced us that if vitamin A were to be prepared in a state approaching purity, it was certainly desirable, if not indeed absolutely essential, to start from highly active oils. In conjunction with Dr J. A. Lovern of the Torry Research Station, Aberdeen, a large number of fish-liver oils of different species have been examined under conditions most favourable to conservation of the vitamin and, as a result, enormous variations in potency have emerged not only between the oils from various species but also between different specimens of oil from a single species. Apparently the potency of some fish-liver oils is much lower in the winter than in summer, but this seasonal variation provides, we believe, only a partial explanation of the wide deviations. From the mass of data in our hands, one fact very soon impressed

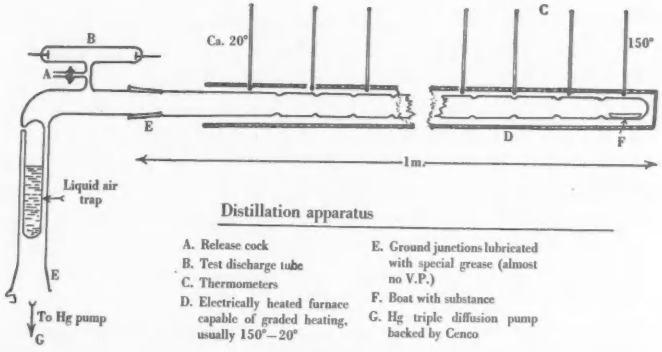
itself, namely that of all fish-liver oils studied, that from the halibut is outstanding in showing occasionally unusually high potency, and it is such oils that we have generally employed as starting material in this investigation. The importance of a careful selection of raw material will be evident from Table I, which illustrates by means of the spectroscopic maxima the wide variations observed in halibut-liver oils.

The general method of saponification adopted by us consisted in heating the oil (500 g.) with 12 % alcoholic potash (1250 cc.) at 75° for 1 hour in an atmosphere of nitrogen under slightly reduced pressure, during which period approximately half the alcohol distilled over. The cooled solution was diluted with distilled water (6000 cc.) and the unsaponifiable matter removed by repeated extraction with ether. After drying over anhydrous sodium sulphate and removal of solvent, the residue was dissolved in hot methyl alcohol (150 cc.) and left to stand, when the bulk of the sterols (mainly cholesterol) present in the mixture separated and were removed at the pump. The filtrate was reduced to half bulk in an atmosphere of nitrogen, and cooled to $-10°$, when more sterol crystallised and was removed. The residual solution was then cooled to about $-50°$ in a mixture of solid carbon dioxide and ether, and after standing for some time rapidly filtered through a jacketed sintered filter maintained at the same temperature. The residue, which had a comparatively low vitamin content, was rejected. The filtrate was diluted largely with water and extracted with light petroleum (B.P. 40–50°) and the solvent finally removed from the dried extract, which was then heated at 70° in a high vacuum. The stages in the concentration of the vitamin, starting from two rich halibut oils (*d*) and (*e*) are reproduced in Table I.

An estimation of the sterol content of (*e*), after treatment at $-50°$, by means of digitonin showed that approximately 3 % was present. Resaponifications of different samples with both sodium ethoxide and sodium methoxide were carried out, but these failed to produce products of increased potency. For the further concentration of the vitamin attempts were made to separate it both by methods involving the distribution between two solvents and also by adsorption on kieselguhr, calcium carbonate and kaolin, but these processes failed to effect any definite increase in potency as measured spectroscopically. It was therefore decided to resort to distillation in a very high vacuum. Attempts to distil vitamin A have previously been reported by Drummond and Baker [1929], who employed pressures near 0·01 mm., but found that under these conditions the vitamin was largely destroyed in the process. In the hope of avoiding this decomposition a special apparatus was constructed (see Fig. 1) whereby fractional distillation of the already very rich concentrates could be carried out at a pressure certainly below 0·0001 mm. The halibut concentrate (10 g.) was introduced into the boat, which was then heated to between 150 and 155°, when distillation commenced. The main fraction, which formed a heavy golden-yellow oil (*c* in Table II) was collected over a period of $3\frac{1}{2}$ hours. A spectrographic examination of this apparently

Table II.

Material	Condensation temp.	Weights of fractions g.	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	580 $m\mu$	328 $m\mu$	
Sterol-freed non-sap. from halibut-liver oil	—	10	2350	1550	1000	
Distillation fractions:						
(*a*)	25–50°	0·14	480 (624 $m\mu$)	400 (567 $m\mu$)		The ultra-violet absorption shows a clear maximum at 285, a pronounced inflexion at 330 and a small inflexion near 371 $m\mu$ $E^{1\%}_{1\,cm.}$ {285 $m\mu$ = 271 330 $m\mu$ = 203
(*b*)	50–80°	0·11	410	340		$E^{1\%}_{1\,cm.}$ {290 $m\mu$ = 250 maximum 328 $m\mu$ = 170 inflexion. There are feeble indications of finer structure in the selective absorption.
(*c*)	80–110°	4·9	1760	1160		The general shape of the absorption curve is similar to the normal vitamin A curve, but more selective absorption is superimposed, sharp maxima occurring at 393, 368, 348, 330, 313·5 and 297 $m\mu$ $E^{1\%}_{1\,cm.}$ 330 $m\mu$ = 710
(*d*)	110–120°	0·57	600	400		The same fine structure is shown $E^{1\%}_{1\,cm.}$ 330 $m\mu$ = 265
(*e*) (semi-solid yellow wax; crystals of cholesterol)	120–140°	0·15	250	160		In addition to an inflexion near 370 $m\mu$, the absorption appears to consist of a fusion of two continuous bands with maxima respectively at 330 and 290 $m\mu$. E in each case approx. 100. Small quantities of substances exhibiting the narrow bands also occur.
(*f*)	140–155°	4·2	180 (610 $m\mu$)	180 (578 $m\mu$)		A very flat maximum occurs at 290 $m\mu$. $E^{1\%}_{1\,cm.}$ = 65, but there is little evidence of the narrow bands



Distillation apparatus

A. Release cock
B. Test discharge tube
C. Thermometers
D. Electrically heated furnace capable of graded heating, usually 150°–20°
E. Ground junctions lubricated with special grease (almost no V.P.)
F. Boat with substance
G. Hg triple diffusion pump backed by Cenco

Fig. 1.

Table III.

Material	B.P.	Time of collection (min.)	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	C.P. blue value	Analysis % C	% H
A. Halibut non-sap. before distillation (12·3 cc.)	—	—	1000	2350	1550	50,000	—	—
Fractions:								
(*a*) 1·5 cc.	120–137°	15	930	2400	1270	53,000	—	—
(*b*) 2·0 cc.	137–138°	12	1190	3000	1600	54,000	—	—
(*c*) 3·7 cc.	138–139°	85	1200	3160	1470	55,000	82·7	11·1
(*d*) 0·75 cc.	139–180°	13	810	2470	1160	50,000	—	—
(*e*) 2·25 cc.	200–225°	30	750	1810	1020	47,000	81·6 81·2	11·5 11·2
(*f*) Solid	225–310°	38	*	—	—	5,500	80·7 80·6	10·4 10·0
B. Concentrate: before distillation (24 cc.)	—	—	1150	3020	1450	57,000	84·2 84·1	11·3 10·9
after distillation, main fraction (12 cc.)	137–138·5°	57	1300	3680	1860	64,000	82·5 82·5	11·1 10·7
C. Another concentrate: before distillation (6·4 g.)	—	—	1100	3250	1950	50,000	—	—
after distillation:								
(*a*) 0·7 g.	130–137°	10	—	—	—	52,000	—	—
(*b*) 2·5 g.	137–138°	15	1300	3710	1850	60,000	82·4	11·0
(*c*) 1·0 g.	138·5–170°	15	—	—	—	54,000	—	—

* Max. stronger than 617 mμ max.

undecomposed distillate revealed that actually readily detectable changes had occurred, for in place of the typical continuous ultra-violet absorption band at 328 mμ an absorption curve showing well defined narrow bands was now exhibited. These bands were also found when the vitamin was dissolved in alcoholic hydrogen chloride and in the material obtained on the regeneration of a vitamin A concentrate from the blue antimony trichloride colour test [Edisbury *et al.*, 1932]. The experiment was repeated with a richer starting material ($E^{1\%}_{1\,cm.}$ 328 mμ = 1150) with similar results; it thus appeared plausible that the molecular rearrangement involved might be due to the prolonged period of heating, possibly combined with some catalytic effect due to the large surface of glass with which the vapour comes into contact. With the very helpful collaboration of Dr F. H. Carr and Mr W. Jewell of British Drug Houses, Ltd., the distillation of rich halibut oil concentrates was also carried out in a new type of molecular still, whereby rapid fractional distillation could be effected at a pressure below 0·00001 mm. Under these conditions the vitamin distils without decomposition or molecular change, giving a main fraction, B.P. 137–138°, having considerably enhanced potency. The results of typical distillations are summarised in Table III. Redistillation of the best fraction resulted in a slight decrease of potency as measured

spectroscopically and it would thus seem that the method had been utilised to the point of maximum efficacy. In order to test this point on different material a sturgeon-liver oil was employed which, although initially not particularly rich in vitamin A, after saponification and freezing provided an unsaponifiable fraction of very high potency. The distillation of this is recorded in Table IV, from which it will be seen that the spectrographic values are in excellent agreement with those obtained from the halibut concentrates.

Table IV.

Material	B.P.	Time of collection (min.)	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	617 $m\mu$	580 $m\mu$	C.P. blue value	Analysis % C	% H
A. Sturgeon-liver oil	—	—	18	39	19	—	—	—
Total non-sap. (4·8 %)	—	—	200	500	290	—	—	—
Non-sap. after treatment at −50° (1 %) (6·2 g.)	—	—	1080	3380	1680	57,000	82·1 82·3	10·7 10·5
After high vac. distillation:								
(1) 1 cc.	120°	15	—	—	—	—	—	—
(2) 1 cc.	120–137°	5	1300	3950	1990	—	—	—
(3) 2·8 cc.	137–138°	12	1330	4200	2130	64,000	82·8 82·6	10·9 10·7
B. Mammalian-liver fat	—	—	235	780	470	11,000	—	—
Non-sap. after treatment at −50° (20 %) (9·2 g.)	—	—	1000	2750	1370	—	82·4	11·1
After distillation: Fraction:								
(1) 2·7 g.	143–149°	10	1250	3620	2350	50,000	82·7	11·1
(2) 1·2 g.	149–150°	10	—	—	—	—	—	—
(3) 0·9 g.	150–165°	10	—	—	—	—	—	—
(4) 4·2 g.	Above 165°	—	—	—	—	4,200	—	—

In order to extend these results it seemed desirable to attempt a concentration of vitamin A from an oil of mammalian source, and we are indebted to Messrs Lever Brothers Ltd. for kindly placing a partially concentrated mammalian extract at our disposal. This was again worked up in the manner already described and gave a highly viscous dark unsaponifiable fraction, the spectrographic data for which (see Table V) were in good agreement with the values obtained from similar fractions of the halibut and sturgeon concentrates. Owing to the very high viscosity of the product the distillation into fractions in this case was rather less sharp, but it will be observed that the main fraction again gives substantially the same values for the 328, 617 and 580 $m\mu$ bands as with the fish-liver distillates. By thus obtaining from totally distinct sources concentrates of constant potency a considerable advance has been made. Certain difficulties still remain before proof of homogeneity in the highly active fractions can be affirmed. In the first place, the evidence

Table V.

Material	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	Analysis % C	% H
Halibut-liver oil non-sap.	1000	2350	1550	—	—
Small scale adsorption exp. (middle layer) (3 g.)	1100	2730	1470	81·3 81·2	10·7 10·5
Large scale adsorption exp. (middle layer) (17·5 g.)	1050	2470	1220	—	—
Halibut-liver oil non-sap. distillate	1300	3710	1850	82·4	11·0
Adsorption of distillate	1350	4350	2350	83·2	10·8
Repeat adsorption	1330	4320	2320	82·9	10·6
Sturgeon-liver oil non-sap.	1000	2500	1250	81·4	10·6
Adsorption (middle fraction)	1100	2850	1470	81·9	10·6
Repeat adsorption	835	2160	1430	81·7	10·9
Sturgeon-liver oil non-sap. distillate	1330	4200	2130	82·8 82·6	10·9 10·7
After adsorption (middle layer)	Fine structure	1950	1600	85·8 85·5	10·6 10·5
Mammalian-liver oil non-sap.	1000	2750	1370	82·4	11·1
After adsorption (middle layer)	1300	3810	1995	83·1	11·0
Repeat adsorption (middle layer)	—	1450	1140	83·1 83·4	10·3 10·4
Mammalian non-sap. distillate	1250	3620	2350	82·7	11·4
Adsorption (middle and bottom fractions)	1320	3920	2500	82·8	10·9

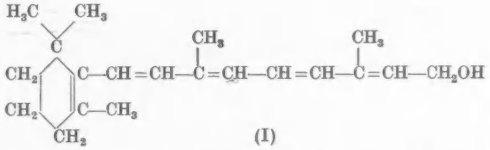
Table VI.

Material		$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	693 mμ	Analysis % C	% H
Halibut non-sap. (best distillate)		1300	3680	1860	370	82·5 82·5	11·1 10·7
Another halibut non-sap. distillate (after adsorption)		1350	4350	2350	900	83·2	10·8
Sturgeon non-sap. (best distillate)		1330	4200	2130	1020	82·8 82·6	10·9 10·7
Mammalian non-sap. (after adsorption)		1300	3810	1995	No band	83·1	11·0
Mammalian non-sap. distillate		1250	3620	2350	No band	82·7	11·4
Prof. Karrer's halibut preparations (Liverpool measurements)	(*a*)	1350	4130	2250	364	83·64 83·8	11·1 10·8
	(*b*)	1250	3250	1600	300	—	—

which has been adduced by Heilbron, Gillam and Morton [1931] concerning the complex nature of the reaction of antimony trichloride remains unshaken, and no satisfactory explanation of the highly selective inhibition of the 617 mμ band, a phenomenon which persists in the richest distillates, is yet forthcoming [Morton, 1932]. Even were this difficulty removed a further source of uneasiness arises in the variable intensity of the absorption band with head at 693 mμ in the colour test. The values for this band for different purified concentrates are summarised in Table VI and whereas with sturgeon oil the band was clearly seen at all stages in the concentration at approximately one quarter of the intensity of the 617 mμ band, with halibut-liver oils its intensity varied according to the starting material from one-tenth to one-quarter of that of the 617 mμ band. In the case of the mammalian concen-

trates, which are otherwise indistinguishable from the richest liver oil concentrates, no selective absorption at 693 $m\mu$ could be observed either in the crude extract or in the purified distillates. It is thus difficult to resist the conclusion that even the richest concentrates so far obtained are liable to contain variable quantities of a substance which is chromogenic towards antimony trichloride but is different from vitamin A itself.

While this work was in progress two papers appeared by Karrer, Morf and Schöpp [1931, 1, 2] in which an account is given of the preparation of a vitamin concentrate which, according to Euler [1931] gives a Carr-Price value of 130,000. The method employed by Karrer and his collaborators for the final concentration of the vitamin consisted in adsorption of the sterol-free unsaponifiable fraction of the liver oils of both halibut and *Scombresox saurus* (Saury Pike) on Merck's specially prepared alumina on the lines of Tswett's chromatographic analysis. The purified product, which the authors consider may be regarded as approximately homogeneous, was obtained as a pale yellow viscous oil which gave analytical results in good agreement with either the formula $C_{20}H_{30}O$ (5 double bonds) or $C_{22}H_{32}O$ (6 double bonds). On catalytic hydrogenation the vitamin was found to take up a volume of hydrogen equivalent to 4·9 mols calculated on a molecular weight of 286. Treatment with ozone gave geronic acid, in amount indicating the presence of one ionone ring, whilst quantitative oxidation with permanganate and chromic acid produced acetic acid in amounts corresponding respectively to 9·7 % and 16·3 % C-methyl. From these results the authors favour formula (I) as probably representing the structure of vitamin A.



(I)

The wide discrepancy between the Carr-Price value recorded by Euler [1931] for the purified vitamin of the above authors and the value of about 65,000 units given for our distilled preparations led us to question whether the distillation method was in reality producing as rich a vitamin A concentrate as that obtained by the chromatographic process of fractionation. We therefore decided to test this method for ourselves, using the Merck "Fasertonerde" employed by Karrer and his colleagues. The apparatus used by us consisted of a long glass tube *T* (Fig. 2) filled to a height of 75 cm. with the fibrous alumina previously dried at 150–200° in a current of carefully purified nitrogen. Nitrogen was first repeatedly drawn through the apparatus so as to ensure complete removal of air. A solution of the selected concentrate (2·5 g. in 25 cc. light petroleum) was then introduced at *S* and the pressure reduced slightly so that on opening the cock t_3 it could be drawn into the adsorbing material. The solution, which penetrated the column to a depth of

about 20 cm. was now slowly washed down the whole column by introducing further quantities of light petroleum into *S*. The apparatus was then disconnected and the tube *T* cut so that the adsorbent was divided into three equal parts. Each section was then separately extracted with hot methyl alcohol (50 cc.), the extract diluted with distilled water (200 cc.) and the vitamin re-extracted with light petroleum.

Table V gives a summary of the results obtained in applying this method of purification to sterol-free vitamin A concentrates. It will be seen that

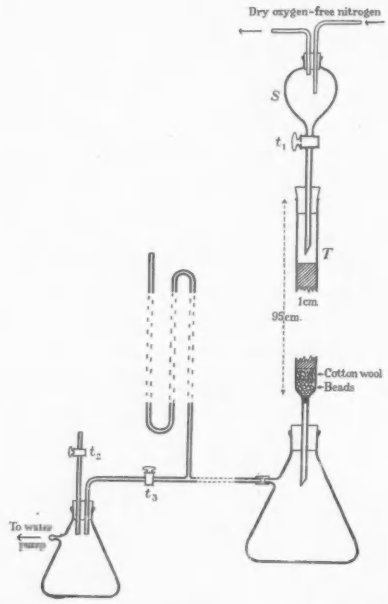


Fig. 2. Apparatus used in the experiments on selective adsorption.

although very definite concentration of the vitamin is effected the results prove that the method possesses no real advantage over that of distillation in a high vacuum. In our experience the method is only applicable to small quantities of material, and further, as measured spectroscopically, the effects of a second fractionation are scarcely perceptible. The increase from 9100 to 10,500 C.L.O. units recorded by Karrer, Morf and Schöpp [1931, 1, 2] as a result of a second adsorption treatment of this concentrate is more probably due to the removal of traces of inhibitors, which, as is well known, greatly modify the blue colour reaction, than to a significant increase in the vitamin A content.

In order to ascertain finally whether the adsorption method was capable

of effecting concentration beyond the highest point attained by distillation we have submitted the main distillates both from the halibut- and the sturgeon-liver oils to the chromatographic process, and have observed either a definite loss of potency or a complete failure to raise the potency beyond the point $E^{1\%}_{1\,cm.}$ $328 m\mu = 1350$. In the case of the richest sturgeon distillate the application of adsorption actually effected an obvious decomposition of the vitamin as evidenced by the appearance of fine structure in place of the smooth $328 m\mu$ band (see Table V). This failure to enrich the product was also confirmed on a mammalian concentrate.

As our experiments on the purification of vitamin A by the chromatographic method failed to account for the discrepancies in the Carr-Price values previously referred to, we approached Prof. Karrer, who very kindly supplied us with two specimens of his richest concentrate. A detailed spectrographic examination of these proved that they do not differ appreciably, except as regards the intensity of the $693 m\mu$ band discussed elsewhere in this paper, from those prepared by us. The spectroscopic magnitudes for the richest vitamin A concentrates together with Prof. Karrer's two preparations are summarised in Table VI, from which it will be seen that the maximum value ($E^{1\%}_{1\,cm.}$ $328 m\mu$) so far obtained is 1350.

Preparation of concentrates from cod-liver oil.

Emphasis has already been laid on the necessity for using highly potent liver oils as starting materials if concentrates of the highest magnitude of potency are required. That these conditions must be strictly observed is well exemplified in Table VII, where the results of applying the adsorption method of purification to a cod-liver oil initially several times as potent as an average specimen are recorded. After freeing the unsaponifiable matter from sterol by cooling to $-50°$ the potency does not exceed one-third of the maximum value. Fractional adsorption on alumina gives in the first separation the fraction of

Table VII.

		$E^{1\%}_{1\,cm.}$				Analysis	
No.	Material	$328 m\mu$	$617 m\mu$	$580 m\mu$	$693 m\mu$	% C	% H
1	Cod-liver oil	4·7	10·4	7·7	—	—	—
2	Total non-sap. (1·4 % approx.)	255	795	425	120	—	—
3	Residue obtained after freezing out sterol at $-10°$	—	940	550	150	—	—
4	Material frozen out at $-50°$	—	350	200	—	—	—
5	Filtrate from (4)	420	1350	800	—	79·6	12·5
6	Fractional adsorption of (5) on fibrous alumina:						
	(*a*) Top portion	—	280	220	—	—	—
	(*b*) Middle portion	400	700	520	105	81·7	10·8
	(*c*) Bottom portion	600	1780	1110	190	—	—
7	Second fractional adsorption using 6 (*c*):						
	(*a*) Middle portion	—	1860	1250	—	79·9	11·0
	(*b*) Bottom portion	—	375	425	—	81·2	10·8

highest potency in the bottom layer of the tube, and even after repetition of the purification process the potency fails to exceed about one-half the maximum value.

Properties of distilled vitamin A.

The distilled product (from all concentrates) forms a pale yellow viscous oil which becomes mobile on warming. It is readily soluble in organic solvents, somewhat more so in methyl than in ethyl alcohol. The vitamin, as obtained after distillation, is unexpectedly resistant to aerial oxidation but is highly susceptible towards acid media [Edisbury *et al.*, 1932]. On standing, however, even in a sealed tube in the dark, a gradual decrease in potency occurs. Thus both with our best materials and that supplied by Prof. Karrer losses up to 10 %, as measured spectroscopically, occurred in less than a month. Analytical data for the highly active distillates and also for the concentrates prepared by the adsorption method where $E^{1\%}_{1\,\mathrm{cm.}}$ 328 $m\mu$ has reached its highest value are given in Table VI, and it will be noted that the results on the whole are in fair agreement with the values required for $C_{20}H_{30}O$. The carbon values of our preparations are somewhat lower than those recorded by Karrer, Morf and Schöpp [1931, 1, 2] or found by us for Prof. Karrer's most active preparation, despite the fact that the absorption data are in outstandingly good agreement. In some cases distillation, while increasing the optically determined potency, seems to effect a slight decrease in the carbon percentage (compare Table III). An unexplained anomalous result was obtained with the sturgeon-oil distillate, which, when an attempt was made to purify it further by adsorption on alumina, gave a resin of low vitamin potency, the carbon value for which, however, was considerably higher than that found for the untreated distillate (Table V). Molecular weight determinations were carried out with the distillate from each species examined according to the Smith and Young modification [1927] of the Rast micro-method and gave as a mean result the value 312, whereas cryoscopic determinations using benzene as solvent gave a mean value of 327. These results are in good agreement with the value of 330 obtained by Karrer by the Rast method, and with the value of 333 calculated by Bruins, Overhoff and Wolff [1931] based on diffusion experiments (mol. wt.: $C_{20}H_{30}O$, 286; $C_{22}H_{34}O$, 314).

Hydrogenation.

Whereas crude vitamin A concentrates are strongly resistant to catalytic hydrogenation, the distilled products can be hydrogenated fairly readily using either palladium or Adams's platinum oxide. The absorption is rapid up to the point when about two-thirds of the total hydrogen is absorbed, after which the rate slows down considerably, and hydrogenation is completed only after many hours and with addition of fresh catalyst. In our experiments we have found that the volume of hydrogen absorbed corresponds to 4·5 molecules, based on $C_{20}H_{30}O$ (5 double bonds) or 4·9 on the less probable $C_{22}H_{32}O$ (6 double bonds) formula. Analysis of the crude hydrogenation pro-

duct gave, in the case of two halibut distillates and the mammalian distillate, the following values respectively: (*a*) C, 79·9; H, 13·3; (*b*) C, 79·8; H, 13·1; (*c*) C, 80·9; H, 12·8 %. In the case of (*b*), fractional distillation in a high vacuum gave a main fraction, B.P. 168–172°/0·06 mm., and a small amount of a higher boiling fraction, B.P. 180–210°/0·08 mm., from which crystalline material separated on long standing. The solid was removed by filtration and after crystallisation from methyl alcohol was obtained as a colourless wax, M.P. 50–53° (Found C, 75·6, 75·8; H, 13·0, 12·9 %). The quantity of this material at our disposal was unfortunately too small to allow of its further investigation but it is our intention to study the hydrogenation of larger quantities of distilled vitamin A so soon as fresh starting material becomes available. The main liquid distillate was tested with tetranitromethane and found still to be slightly unsaturated, as evidenced by the production of a definite yellow colour. It was accordingly rehydrogenated in acetic acid solution with Adams's platinum oxide, when a further quantity of hydrogen was absorbed. The product was again distilled in a high vacuum, and distilled almost wholly at 158–163°/0·04 mm. No colour was given with tetranitromethane, and the product, which was obtained as a thick colourless oil, was again analysed. (Found: C, 81·3; H, 13·0; $C_{20}H_{40}O$ requires C, 81·0; H, 13·6 %.)

Iodine values.

A series of iodine values was determined according to Rosenmund and Kuhnhenn's method [1923] and gave 306, 315, 324 on three different samples. (Iodine value for $C_{20}H_{30}O$ (5 double bonds), 448.) It would seem from these results that the reagent attacks the ethylene linkage of the ionone ring only extremely slowly if at all. Evidence in favour of this assumption may be adduced from experiments on β-ionone when the following results were obtained: (*a*) 5 min. contact with Br reagent 198, 190, (*b*) 20 min. 190, 196, (*c*) 60 min. 231, 234. (Iodine value for $C_{13}H_{21}O$ (2 double bonds), 264.)

Preparation of esters.

Karrer, Morf and Schöpp [1931, 2] have described the preparation of a *p*-nitrobenzoate and an acetate of vitamin A, neither of which is recorded as having been obtained crystalline. We have ourselves prepared a benzoate by the following method. 15 g. of halibut concentrate were dissolved in pure dry pyridine (25 g.) and freshly distilled benzoyl chloride (14 g.) slowly added to the solution. After standing at room temperature for some time, the mixture was heated for 1 hour on a water-bath at 80° in an atmosphere of nitrogen. After cooling, the solution was diluted with water, extracted with ether, and the ethereal extract repeatedly washed successively with dilute sodium carbonate solution and water, and dried over anhydrous sodium sulphate. The deep yellow, thick oil obtained after removal of solvent was repeatedly extracted with hot methyl alcohol until the extracts were no longer coloured. The residual oil (6·2 g.) was taken up in ether, dried, and after removal of

solvent subjected to high vacuum distillation when, as shown in Table VIII, a main fraction was collected distilling between 188 and 198° without noticeable decomposition. The distilled benzoate formed a pale yellow viscous oil which could not be induced to crystallise. (Found: C, 82·2; H, 9·2 %. $C_{20}H_{29}O.COC_6H_5$ requires C, 83·0; H, 8·7 %.) Attempts have also been made to prepare solid esters using dinitrobenzoyl chloride, diphenic anhydride, phthalic anhydride and the acid chloride of anthraquinone-1-carboxylic acid, without success. Further experiments are now being carried out on these lines in the hope of obtaining solid derivatives of the hydrogenated alcohol or its oxidation products.

Table VIII.

Material	Temp. of distillation	Time of distillation, min.	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	Carr-Price blue value	Analysis % C	% H
Halibut-liver oil concentrate used	—	—	1150	3020	1450	57,000	84·2	11·3
Total benzoylated product	—	—	710	1720	860	—	82·1 82·3	8·7 9·0
Benzoylated product (insol. in methyl alcohol) (6·2 g.)	—	—	750	1920	910	40,000	—	—
After high vacuum distillation: Fraction:								
(1) 0·2 g.	165–180°	—	Quantity too small for full examination					
(2) 1·5 cc.	170–190°	20	780	2020	1010	34,000	—	—
(3) 2·5 cc.	195–198°	60	780	2250	1200	37,000	82·2	9·2
(4) 0·3 g.	Over 200°	—	290	550	345	15,000	—	—
(5) 0·7 g. residue	Not volatile at 300°	—	145 (300 at 280 mμ)	105	160	—	—	—

Ozonisation.

We have repeated the ozonisation experiments described by Karrer, Morf and Schöpp [1931, 1, 2] using a halibut-liver oil distillate. Working under the conditions specified by the above authors we have isolated geronic acid in the form of its semicarbazone, which after recrystallisation from ethyl acetate was obtained in colourless needles, M.P. 163–163·5°. The isolation of this acid fully confirms the presence of the ionone ring in the vitamin A structure [see also Heilbron, Morton and Webster, 1932].

Biological potency of the vitamin concentrates.

The physiological potency of certain of the distillates was tested by administration of measured amounts to young rats which had been fed upon a basal diet deficient in vitamin A until growth had ceased. The composition of the ration was

Purified caseinogen	...	...	...	20
Rice starch	...	...	...	80
Salt mixture	...	...	...	5
Dried yeast	...	...	...	5

Vitamin D was supplied by the separate administration of 0·001 mg. of a tested preparation of irradiated ergosterol twice weekly. Both this supplement and the fractions being tested for vitamin A potency were given in the form of solutions in freshly-distilled ethyl laurate. The test solutions of vitamin A concentrates were made up twice weekly and were stored at low temperature in the dark.

The concentrate prepared from halibut-liver oil and fractionated by distillation ($E^{1\%}_{1\,cm.}$ $328\,m\mu = 1300$) promoted growth in doses of $0{\cdot}1\gamma$ daily and cured xerophthalmia in 48 hours (Fig. 3). Smaller doses of this concentrate were not tested but in the case of the distilled concentrate from the sturgeon-liver oil an improvement in general condition and a slow resumption of growth occurred when daily doses as low as $0{\cdot}025\gamma$ were given. A dose of $0{\cdot}05\gamma$ of this material effected a rapid cure of the eye disease in one rat (Fig. 4). These materials would appear to have a higher biological activity than those tested

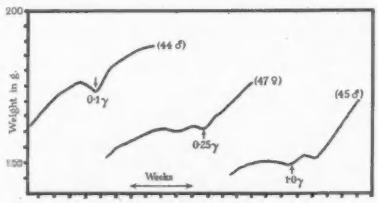


Fig. 3.

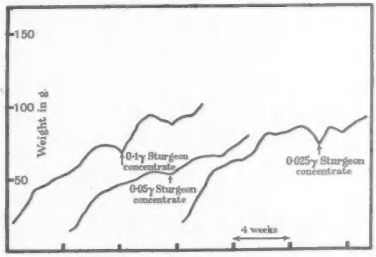


Fig. 4.

Fig. 3. Biological tests on main fraction obtained by distillation of Halibut-liver oil concentrate.
Fig. 4. Showing recovery of growth caused by administration of concentrate of vitamin A prepared from sturgeon-liver oil. Basal diet throughout deficient in vitamin A.

by Euler [1931], Karrer, Morf and Schöpp [1931, 1, 2] and Euler and Karrer [1931], although the differences may to some extent be due to different methods of conducting the feeding experiments.

Discussion.

The position which has now definitely been reached is that the richest products obtained both by us and by Karrer and his collaborators, from mammalian and various fish-livers, are qualitatively and quantitatively indistinguishable in respect of ultra-violet absorption. If the products are not homogeneous, then either the non-vitamin material is relatively diactinic, or the preparations contain substances so closely alike that both exhibit the $328\,m\mu$ band. The absorption spectrum affords no conclusive criterion of homogeneity although it would be indeed curious if exactly the same proportion of material exhibiting negligible absorption were present in the products derived from various species of fish. On the other hand, the discovery of isomeric α- and β-carotenes, both transformed *in vivo* into vitamin A-like

substances [Kuhn and Brockmann, 1931] suggests plainly that strict homogeneity must be demonstrated rather than assumed.

If the ultra-violet absorption spectrum leaves the question of purity an open one, the antimony trichloride colour test is equally indecisive. Even if the difficult choice concerning the origin of the 617 and 583 $m\mu$ maxima can be resolved by the demonstration (which is yet to seek) that both bands are traceable to one entity, the existence of the maximum at 693 $m\mu$ and especially (as shown in Table VI) its variable intensity in different preparations, remains wholly unexplained.

Our own observations in general are at least as consistent with Prof. Karrer's tentative structure (I) for vitamin A as with any alternative, and indeed the weight of evidence is impressively though not conclusively in its favour.

From the standpoint of analytical data, the failure to prepare crystalline derivatives of vitamin A makes it very difficult to compel assent to any claims to having prepared the vitamin in a state of purity. The experimental data on molecular weights lead to a value of 320 ± 15 as against 286 on the basis of the formula. In itself, this small discrepancy is not necessarily significant, but when the results of a very large number of ultimate analyses uniformly indicate variable values not quite consistent with the $C_{20}H_{30}O$ formula, doubts concerning purity are strengthened. Finally, our experiments on fully hydrogenated preparations have provided definite evidence of some contaminant in the resulting product.

The analytical data show that the question at issue is not, however, one of gross impurity, and further the extinction coefficient of the 328 $m\mu$ band is of the correct order to be expected from the proposed constitution. In this connection it must be borne in mind that neither C and H percentages nor molecular weights would be affected by heterogeneity arising out of isomeric forms.

As far as can be judged, a small quantity of an alcohol, probably more saturated than vitamin A, is extremely difficult to eliminate completely when reasonably large quantities of material are under investigation. This probably accounts for the failure to reach the highest potency when the full technical resources are applied to cod-liver or poor grade halibut-liver oils.

Once the remaining doubts concerning homogeneity are resolved, biological, spectroscopic and colorimetric standards can be set up in terms of which the results of various methods of assay can be expressed.

We desire to express our thanks to the Department of Scientific and Industrial Research for grants to two of us (R.N.H. and E.T.W.) in aid of this investigation. We are also deeply indebted to Dr J. A. Lovern of the Torry Research Station, Aberdeen, for the great help he has given us in the collection of fish-liver oils, to Messrs Lever Brothers, Ltd., for placing at our disposal samples of mammalian concentrates, to Dr F. H. Carr and Mr W. Jewell of

British Drug Houses, Ltd., for carrying out the vitamin distillations, and to Mr W. Doran and Mr W. F. Boston of Liverpool University for carrying out the numerous micro-analyses recorded in this paper. Part of the expenses of this investigation was defrayed by a grant from the Medical Research Council, for which we express our thanks.

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Note added August 16th, 1932. Since this paper was written we have received (7. vi. 32) a fresh halibut oil the vitamin A content of which was approximately double that of the richest oil (*e* in Table I) previously examined. The following table shows the potencies at various stages in the extraction of the vitamin. Although the original oil was phenomenally rich in vitamin A (estimated at 10 % vitamin) the final product was not appreciably different from the values recorded in Table VI.

	$E^{1\%}_{1\,cm.}$				Analysis	
Material	328 $m\mu$	617 $m\mu$	580 $m\mu$	693 $m\mu$	C %	H %
Halibut-liver oil	164	430	260	50	—	—
Non-sap., filtrate	—	4000	2000	410	82·4	10·6
− 60°	—	—	—	—	82·7	10·5
Ditto after adsorption						
(i)	—	3950	2100	560	—	—
(ii)	1350	4520	2360	270	83·3	10·7
Distillate, B.P. 136°	1370	4650	2550	450	83·3	11·0

CXL. THE STRUCTURE OF VITAMIN A.

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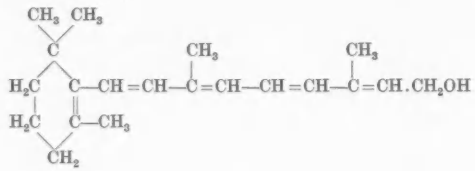
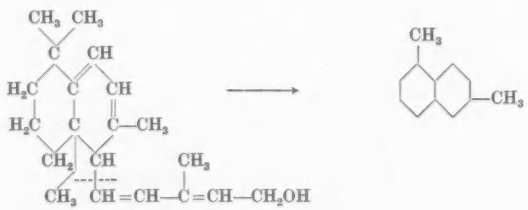
(*Received May 17th, 1932.*)

It has already been shown [Edisbury *et al.*, 1932] that it is possible to obtain by several methods, and especially with acid reagents, a product from vitamin A exhibiting highly characteristic narrow absorption bands. On the basis of the structure (I) suggested by Karrer, Morf and Schöpp [1931] for the vitamin it seemed to us that the new product was probably formed by intramolecular condensation whereby a hydronaphthalene (II) might result.

(I)



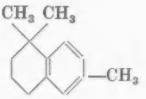
(II)

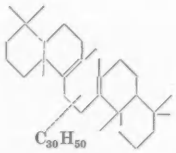


As is well known, many hydronaphthalene compounds can readily be dehydrogenated by sulphur, or better by the Diels method employing selenium, to yield true aromatic naphthalene hydrocarbons. Thus cadalene (1 : 6-dimethyl-4-*iso*propylnaphthalene) or eudalene (1-methyl-7-*iso*propylnaphthalene) is formed from many bicyclic sesquiterpenes [Ruzicka *et al.*, 1922].

On the other hand, ionene (III) [Ruzicka and Rudolf, 1927] and tetra-*cyclo*squalene (IV) [Heilbron and Wilkinson, 1930; Ruzicka *et al.*, 1932], both of which contain *gem*-dimethyl groupings attached to quaternary carbon atoms,

yield on dehydrogenation 1 : 6- and 1 : 2 : 5-trimethylnaphthalene respectively.

(III) 

(IV) 

On the basis of this, we anticipated that the suggested hydronaphthalene derivative (II) would yield with selenium either one or other of the above methylnaphthalenes. On submitting this idea to experimental test we have found that on treatment of the product obtained from a rich concentrate by the action of alcoholic hydrochloric acid with selenium at 300–330°, a good yield of 1 : 6-dimethylnaphthalene results.

The extraordinary ease with which vitamin A is transformed into a cyclised product prompted us to attempt the dehydrogenation with the vitamin itself, with the result that an equally high yield of 1 : 6-dimethylnaphthalene was obtained. It is thus evident that the intramolecular change is due either to the elevated temperature at which the reaction is carried out [cf. Heilbron *et al.*, 1932] or to the action of hydrogen selenide, the presence of which can be observed even at comparatively low temperatures.

Experimental.

A vitamin A concentrate, $E^{1\%}_{1\,cm.}$ 617 $m\mu$, 3140, (7·4 g.), mixed with finely powdered selenium (7·4 g.), was heated under a long air condenser at 300–330° for 48 hours. The cooled viscous product was repeatedly extracted with boiling ether, the combined extracts were filtered and the solvent was removed. The residual oil was then distilled over metallic sodium, whereby the following fractions were obtained.

A. B.P. 120–200°/20 mm. (1·6 g.); redistillation of this fraction over sodium yielded a colourless oil, B.P. 70–110°/5 mm. (1·2 g.).

B. A high boiling fraction, B.P. 200–300°/20 mm. This fraction was obtained as a viscous orange oil which rapidly darkened on standing (1 g.).

1 : 6-*Dimethylnaphthalene.* A warm solution of oil (A) was treated with an alcoholic solution of picric acid (1 g. in 15 cc.), when a slight darkening in colour was immediately observed. On standing the liquid solidified almost completely yielding orange crystals (1 g.) M.P. 100–110°. After recrystallisation from absolute alcohol long orange needles (M.P. 113–114°) were obtained. (Found: C, 56·0; H, 4·2: $C_{18}H_{15}O_7N_3$ requires C, 56·1; H, 3·9 %.) A mixed melting-point with authentic 1 : 6-dimethylnaphthalene picrate (M.P. 112–113°) gave no depression. The hydrocarbon was obtained by shaking an ethereal solution of the picrate with aqueous ammonia. Its absorption spectrum was compared with that of pure 1 : 6-dimethylnaphthalene (Fig. 1) and found to be qualitatively and quantitatively identical.

The *styphnate* was also prepared and forms yellow needles, M.P. 120°. (Found: C, 53·8; H, 3·5. $C_{18}H_{15}O_8N_3$ requires C, 53·9; H, 3·7 %.)

The high boiling fraction (B) failed to give any crystalline derivative with either picric or styphnic acid.

Cyclisation of vitamin A. This was carried out by treating the vitamin A concentrate (11 g.) in alcohol (500 cc.) with concentrated hydrochloric acid (6 cc.). The solution was allowed to stand for 20 minutes at room temperature during which time the pale yellow colour changed to dark brown. Solid sodium carbonate was added until the solution reacted alkaline, when the colour immediately reverted to light yellow. Excess sodium carbonate was filtered off, and the alcohol removed under reduced pressure. The residue was taken up in light petroleum and the petroleum extract repeatedly washed with water. After drying and removal of solvent a light brown viscous oil remained which on spectroscopic examination was found to exhibit well defined absorption bands at 392, 369, 350 and 333 $m\mu$. Treatment of this product with selenium by the method already described above gave 1 : 6-dimethylnaphthalene in a yield of approximately 3·5 %.

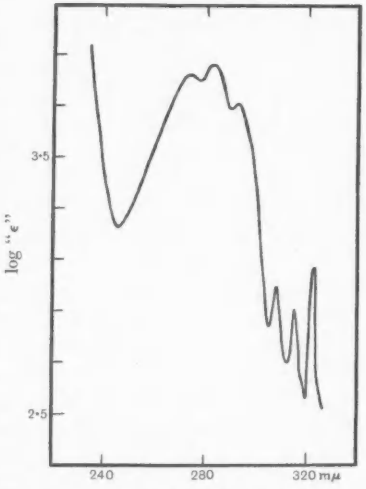


Fig. 1. 1: 6-Dimethylnaphthalene in hexane.

The isolation of 1 : 6-dimethylnaphthalene in good yield by the dehydrogenation of vitamin A definitely establishes the terpenoid nature of the vitamin and the presence in the richest concentrates of a material possessing a constitution which, as far as the fourteenth carbon atom, must be identical with the constitution (I) advanced by Karrer, Morf and Schöpp [1931] for vitamin A.

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CXLI. THE USE OF 7-METHYLINDOLE IN THE ANTIMONY TRICHLORIDE COLOUR TEST FOR VITAMIN A.

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(*Received May 17th, 1932.*)

It is now generally agreed that the blue colour which results when antimony trichloride is added to chloroform solutions of liver oils and concentrates owes its origin, in part at least, to vitamin A. As a quantitative criterion of potency, the test, however, suffers from considerable disadvantages, among which the following may be mentioned.

(*a*) The chromogenic power of the substances present in liver oils is smaller than would be expected from the values obtained with the unsaponifiable extracts [Smith and Hazley, 1930; Smith, 1931; Coward, Dyer, Morton and Gaddum, 1931] owing to the occurrence of inhibitors in the saponifiable portion [*cf.* Norris and Church, 1930, 1, 2]. This effect is highly variable and militates powerfully against accuracy in the colorimetric assay [Lovern, Creed and Morton, 1931; Gillam and Morton, 1931].

(*b*) The coloured solutions exhibit complex and qualitatively variable absorption spectra [Morton, Heilbron and Thompson, 1931; Heilbron, Gillam and Morton, 1931]. Maxima in the spectral absorption curves have been recorded at 572–583, 606–620, 643 and 693 $m\mu$, and there is considerable evidence of complexity in the chromogenic constituents of liver oils.

(*c*) The intensity of absorption at a given wave-length or the absolute depth of blue colour varies considerably with the time elapsing between mixing and recording intensities, and with concentrations in the system: $CHCl_3$—$SbCl_3$—vitamin A—non-chromogenic substances.

It has been shown [Heilbron, Gillam and Morton, 1931] that with many oils the spectral absorption curve shown after reaction with antimony trichloride has occurred consists of a summation of two distinct absorption bands with maxima at 572 and 606 $m\mu$ respectively. Sometimes one, sometimes the other, is the more intense. In all cases the 606 $m\mu$ band eventually becomes the stronger, either by spontaneous ageing or treatment with ozone. Increases in the 606 $m\mu$ band are never at the expense of a reduction in the intensity of the 572 $m\mu$ band, which remains practically constant, as also does the 328 $m\mu$ ultra-violet maximum characteristic of vitamin A. In concentrates, the 606 $m\mu$ band is shifted to 617 $m\mu$ and is always much stronger than the other band. Heilbron, Gillam and Morton [1931] were able to show

that in all cases the shorter wave maximum is present in concentrates at 583$m\mu$ but that its existence is usually indicated only by an inflexion in the absorption curve. By employing a slight modification of the ordinary procedure in carrying out the colour test they were able to demonstrate the real existence of this maximum. The preliminary addition of a very small quantity of antimony trichloride reagent to the chloroform solution of the concentrate, one minute before carrying out the ordinary test, effects a striking reduction in the intensity of the blue colour. This reduction is largely confined to the absorption band at 617$m\mu$ so that the 583$m\mu$ maximum, which is masked in the solution obtained by the ordinary procedure, is now fully revealed.

In discussing the results, the authors envisaged two main possibilities, namely that the two chromogenic mechanisms denoted either two separate chromogens or two distinct reactions between vitamin A and antimony trichloride.

Brode and Magill [1931] also observed the existence of two variable absorption bands in the colour tests, and recorded maxima at 608 and 578$m\mu$. The solutions were unstable and gradually became red, exhibiting maxima at 532 and 472$m\mu$. These authors also correlated the 608 and 532$m\mu$ maxima and considered the latter to arise from a spontaneous decomposition of the substance causing the 608$m\mu$ band. Similarly the 578 and 472$m\mu$ maxima were found to be connected in some way. In this laboratory the 532 and 472$m\mu$ maxima have not been regularly noticed and little evidence of any significant correlation has been obtained. It is, however, important that Brode and Magill obtained valuable evidence of a complex chromogenic mechanism. Like Heilbron, Gillam and Morton [1931] they were however unable to decide whether liver oils contain two separate chromogens or whether the two bands indicate alternative or successive reactions between vitamin A and antimony trichloride.

Another valuable piece of confirmatory evidence was supplied by Emmerie, Eekelen and Wolff [1931]. These authors state: "By treating a vitamin A preparation...with some drops of furan, methylfuran, pyrrol, indol, or skatol (all substances of related chemical structure), and then adding antimony trichloride, the mixture turns purple instead of blue, and in the spectroscope the 610$m\mu$ band is no longer seen, while the 572$m\mu$ band remains unaltered. The physiological activity is unimpaired and the preparation (without antimony trichloride) shows the 328$m\mu$ band just as the original substance did."

Eekelen *et al.* [1931] extended these observations and recorded data which tend to increase the difficulties of interpretation. Apparently, the low values in the colour test for oils are due to the presence of traces of inhibiting substances, but the results, whilst far from conclusive, point rather to heterogeneity of chromogens than to different stages in the reaction between antimony trichloride and vitamin A.

The work of the Dutch investigators seemed to indicate a more convenient method for separating the chromogenic effects than the original one emanating

from these laboratories. Accordingly the statements made were tested and fully substantiated.

A large number of pure substances of the type suggested were tried, including 2-, 3- and 7-methylindole. The most convenient substance was found to be 7-methylindole.

In Table I the fact is demonstrated that the 617 $m\mu$ maximum suffers considerable inhibition before the 583 $m\mu$ maximum is appreciably affected. In

Table I. *Effect of 7-methylindole on the antimony trichloride colour test applied to materials very rich in vitamin A.*

	Wt. of concentrate used mg.	Wt. of 7-methyl-indole used mg.	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	$E^{1\%}_{1\,cm.}$ 583 $m\mu$	Ratio wt. 7-methyl-indole/wt. concentrate
1	0·0562	0·375	660	1070	6·5
	,,	0·300	750	1100	5·2
	,,	0·225	910	1180	3·9
	,,	0·150	1250	1250	2·6
	,,	0·075	1525	1310	1·3
	,,	0	2350	1310	—
	,,	Trace only	—	1300	—
2	0·0522	0·375	690	965	7·18
	,,	0·3	790	960	5·76
	,,	0·225	1206	1200	4·3
	,,	0·150	1300	1210	2·88
	,,	0·075	1870	1260	1·44
	,,	0	2160	1350	—
3	0·05	0	3300	1600	—
	,,	0·2	1600	1550	4
4	0·04	0	4200	2135	—
	,,	0·16	2000	1980	4

Table II the last column indicates the intensity at 583 $m\mu$ after just sufficient 7-methylindole has been added to render the 617 $m\mu$ maximum definitely weaker than the 583 $m\mu$ maximum. The main results may be summarised as follows.

(1) 7-Methylindole, at the concentrations used, is practically transparent in the region 290–350 $m\mu$.

(2) The absorption spectrum of a solution containing vitamin A concentrate and 7-methylindole in chloroform agrees quantitatively with the summation of the separate curves for the concentrate and the 7-methylindole, so that there is no evidence of chemical interaction before the addition of antimony trichloride.

(3) In all cases, from cod-liver oils to the richest concentrate, the incorporation of a trace of 7-methylindole effects an inhibition of the capacity to give the blue colour.

(4) The inhibition can be regulated by adding greater or smaller quantities of 7-methylindole.

(5) The masking effect of the 617 band on the 583 $m\mu$ maximum can be eliminated by adding a carefully regulated quantity of inhibitor so that the

Table II.

Material	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ Ultra-violet absorption	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ Antimony trichloride colour test without added 7-methylindole	$E^{1\%}_{1\,cm.}$ 583 $m\mu$ Antimony trichloride colour test without added 7-methylindole	$E^{1\%}_{1\,cm.}$ 583 $m\mu$ (colour test) intensity when just enough 7-methylindole has been added to make 617 $m\mu$ band weaker than that at 583 $m\mu$
Halibut-liver oil non-sap.	1000	2350	1350	1300
(average)	1154	3030	1450	1300
Pig-liver concentrate	525	1200	840	650
Cod-liver oil concentrate	270	541	360	315
Mammalian concentrate	130 *(290, 190)	320	230	200
Cod-liver non-sap.	115	221	124	110
,, ,,	57·5	96	65	60
Pig-liver oil (*a*)	30	63	41	34
,, (*b*)	14·4	36·8	17·5	14·8
Pig-liver oil (store pigs) (*a*)	—	0·99	0·92	0·9
,, ,, (*b*)	—	1·6	1·1	1·0
Cod-liver oil crude non-sap.	16 *(290, 14)	34·4	18	16
Sheep-liver oil	19·7 *(290, 25·7)	30	28	28
Cod-liver oil	0·73	0·76	0·59	0·5
,,	5·5	15·2	9·0	8·2

* Occasionally in fish-liver oils and concentrates, and more often in materials from mammalian livers, we have observed an absorption band (max. 290 $m\mu$) superposed on the 328 $m\mu$ band of vitamin A. Dr T. Moore and Dr N. S. Capper have also observed this effect (private communication). At first sight this band would seem to be due to the decomposition product with a maximum at 290 $m\mu$. This explanation is not wholly satisfactory because the materials exhibiting a maximum at 290 $m\mu$ without other evidence of decomposition, exhibit high values in the colour test for the 583 $m\mu$ band. A substance closely related to vitamin A and not derived from it by decomposition may be responsible. In this connection it is well to emphasise a point which merits further investigation: as has been shown by Kuhn and Brockmann [1931], α-carotene and β-carotene, containing α- and β-ionone rings respectively, are both capable of inducing growth. Presumably each is converted *in vivo* into a vitamin A-like substance. There is thus the possibility of two isomeric vitamin A compounds, one with and one without the ethenoid linkage of the ring conjugated to the side chain. The properties of such isomerides cannot be predicted but it is quite possible that the 290 and 328 $m\mu$ bands may respectively be due to the α- and β-isomerides.

617 and 583 $m\mu$ bands can be seen side by side, at approximately equal intensities, before any appreciable inhibition of the 583 $m\mu$ band has occurred.

(6) Using the richest vitamin A concentrates (Carr-Price blue value $> 50{,}000$), the 617 $m\mu$ band is almost exactly twice as intense as the 583 $m\mu$ maximum without addition of inhibitor, but if 7-methylindole is added before the antimony trichloride reagent, in the ratio 7-methylindole/concentrate = 4/1, the two bands appear with roughly equal intensity, the inhibition being confined to the longer wave maximum.

(7) The addition of larger quantities of 7-methylindole effects a slow inhibition of the 583 $m\mu$ band, but this is always very much smaller than the inhibition of the 617 $m\mu$ maximum.

(8) In carrying out the antimony trichloride colour test as a quantitative measure of vitamin A potency, serious inaccuracies arise when the test is carried out on oils owing to the variable action of inhibitors, but these are greatly reduced when observations are carried out on the non-saponifiable

matter [Smith and Hazley, 1930; Coward *et al.*, 1931]. In all cases, much more accurate results can be obtained if attention is concentrated on the 583 $m\mu$ maximum, using just sufficient 7-methylindole to allow the 617 and 583 $m\mu$ bands to be seen in the spectroscope. Under these circumstances, the estimates of potency are in close agreement with the values deduced from the intensity of the 328 $m\mu$ band.

(9) Accepting the molecular weight of vitamin A as near 300 (7-methylindole, 133) and making use of the fact that four parts by weight of inhibitor to one of the richest vitamin preparations are necessary for optimum results, this is equivalent to approximately 10 molecules of 7-methylindole to 1 mol. vitamin.

(10) Inhibition by means of 7-methylindole does not readily occur with the 693 $m\mu$ band recorded by Morton, Heilbron and Thompson [1931], and it does not appear to be possible to establish any rigid correlation between this band and the 583 $m\mu$ maximum.

(11) In view of the fact that inhibition occurs with complete success when the colour tests on the richest concentrates of vitamin A are carried out in the presence of 7-methylindole, such concentrates cannot be accepted as homogeneous without a satisfactory explanation of the apparent independence of the 617 and 583 $m\mu$ bands. Vitamin A may well react in two stages with antimony trichloride, but there is no direct evidence on this point. The ultraviolet absorption spectrum discloses definite information of only one absorbing entity, the colour test reveals two chromogenic mechanisms. No decision is yet possible as to whether the richest concentrates are mixtures of extremely closely related substances, or whether one and the same molecule can give rise to the complex colour reaction.

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CXLII. THE IMMUNOLOGICAL PROPERTIES OF ALKALI-TREATED PROTEINS.

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(*Received June 26th, 1932.*)

The influence of acid and alkali on proteins in relation to the antigenic properties of these proteins has been studied by many authors. Wells [1909] noted the marked loss of antigenic power when crystalline egg-albumin was treated with sodium hydroxide, whereas little loss occurred following similar treatment with hydrochloric acid. Ten Broeck [1914] found that racemised egg-albumin (prepared by Dakin's [1912] method) was non-antigenic, and similar results with other proteins were obtained by Landsteiner and Barron [1917] and by Kahn and McNeal [1918].

Evidence of restoration of antigenic properties to alkali-treated proteins was furnished by Landsteiner and Barron [1917], who showed that when the non-antigenic alkali-treated serum-proteins were treated with nitric acid, antigenic xanthoproteins were formed; these xanthoproteins prepared from alkali-treated proteins gave precipitin and complement-fixation reactions with antisera to xanthoproteins from untreated serum-proteins and also acted as true antigens when injected into rabbits. These results, indicating some reversal of the process or processes which originally caused loss of antigenic power, are very significant, and it is possible that a further study of this phenomenon would add considerably to our knowledge of protein antigens.

In view of the importance of this relationship between the action of alkali and the loss of antigenic properties, it was decided to attempt to define more precisely the relationship between p_H and loss of antigenicity, and also to try to obtain, by some method other than that used by Landsteiner and Barron [1917], confirmatory evidence of the restoration of antigenic power to alkali-treated proteins. For the latter purpose, it was considered that iodination of the alkali-treated proteins would be most suitable, since iodination can be effected in neutral or faintly alkaline solution, and, although it replaces the original species specificity of serum proteins by a specificity determined principally by the 3 : 5-diiodotyrosine groupings [Wormall, 1930], iodination does not lead to any appreciable loss of antigenic properties.

In the investigation described here, the proteins of horse-serum were rendered non-antigenic by treatment with sodium hydroxide for approximately 24 hours at 18° in one series and 30° in another series. Fractions of these "alkali-proteins" were nitrated and others iodinated, and precipitin

and complement-fixation tests were then carried out with these preparations to determine whether the power to react with various antisera had been restored by nitration and iodination. These protein preparations, the alkali-treated proteins and the corresponding iodinated and nitrated alkali-treated proteins, were also tested for antigenic power by injection into rabbits, precipitin and complement-fixation tests being made at intervals.

Experimental.

Antigens used for injection.

The following antigens were prepared for immunisation purposes. NaCl to give 0·9 %, and phenol to give 0·25 % were added to all the antigen solutions, except those containing iodoprotein, which received NaCl only. All the solutions were kept in the ice-chest.

NaOH-treated serum-proteins. (*a*) *At* 18°. A mixture of 1500 cc. of horse-serum, 235 cc. of 7·4 *N* NaOH and 15 cc. of toluene was kept at 18° for 22 hrs. Conc. HCl was added to give maximum precipitation, and the precipitated proteins were filtered off and washed well with water; they were then dissolved in water with the addition of a small amount of *N*/2 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution with p_H about 7·8. NaCl and phenol were then added in the amounts given above and the solution kept in the ice-chest. The final volume was 1000 cc. and the solution contained 5·75 % of protein (solution *A*).

(*b*) *At* 30°. A mixture of 2500 cc. of horse-serum, 225 cc. of 12·15 *N* NaOH and 25 cc. of toluene was kept in an incubator at 30° for 24 hrs. The proteins were precipitated by HCl as above, and finally obtained in solution (800 cc.) at about p_H 7·8. The solution contained 5·6 % of protein (solution *B*).

Iodinated NaOH-treated proteins. (*a*) 250 cc. of the solution of NaOH-treated proteins prepared as above (solution *A*) were mixed with 250 cc. of distilled water and 250 cc. of 7 % NH_4OH solution, and treated with *N*/10 iodine solution until there was a considerable excess of iodine [*cf.* Wormall, 1930]. The iodinated proteins were precipitated by the addition of 10 % acetic acid to give maximum precipitation, and the precipitated protein was removed by centrifuging. This precipitate was washed with water and dissolved in water with the addition of sufficient 10 % Na_2CO_3 solution to give a clear solution, the reaction of the solution being adjusted to p_H 7·6. 250 cc. of a solution containing 5·2 % of protein were obtained in this manner.

(*b*) 250 cc. of the solution obtained by treating horse-serum with NaOH at 30° (solution *B*) were iodinated as above.

Nitrated NaOH-treated proteins (*xanthoproteins*). The method used for nitration was that involving the action of conc. HNO_3 at 18° in the presence of urea [*cf.* Landsteiner and Prášek, 1913]. The products of this reaction react immunologically like proteins nitrated by a method which does not involve treatment with strong acid, *viz.* the tetranitromethane method [Wormall, 1930], and in this paper the terms xanthoprotein and nitrated protein are used synonymously.

(*a*) To 300 cc. of a solution of NaOH-treated proteins (solution *A*) a few grams of urea were added followed by 600 cc. of conc. HNO_3 slowly with constant stirring and cooling. The mixture was kept for 45 mins. at 18° and then poured into a large volume of distilled water. The precipitate was then filtered off, washed with water and dissolved in water with the addition of a little *N*/5 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution. The p_H was adjusted to 7·8 and 300 cc. of a solution containing 3·5 % of protein were obtained.

(*b*) 300 cc. of the protein solution obtained by NaOH-treatment at 30° (solution *B*) were nitrated in a similar manner.

Immunisation.

Each antigen solution was tested on four rabbits, the injections being made weekly. Serum from each rabbit was tested 7 days after the third and each subsequent injection, *i.e.* just before the fourth, fifth, sixth and seventh, and, in certain instances, the eighth injection, and 7 days after the final injection.

Series (*a*). For the first three injections, an amount of the solution (solution A or the corresponding iodinated or nitrated protein solution) which contained approximately 0·25 g. of protein (4·3 cc. to 7 cc.) was injected intravenously into each rabbit. Five further injections of a volume of the solution which contained approximately 0·35 g. of protein were made intraperitoneally.

Series (*b*). With solution B and the corresponding iodinated and nitrated protein solutions each rabbit received at each injection a volume of the solution which contained 0·25 g. of protein; each animal had seven injections, the first three or four being intravenous and the rest intraperitoneal.

Precipitin tests.

Two, and, in a few tests, three drops of the immune serum were added to 0·25 cc. of the diluted antigen solution in a small test-tube ($2'' \times \frac{1}{4}''$). The mixture was shaken and observed after it had stood 5 mins., 30 mins., 1 hr. and 3 hrs. at room temp. (16–20°) and after a further 18 hrs. in the ice-chest. The amount of protein in each antigen solution had previously been determined, and the various antigen dilutions were made to correspond to the following dilutions of a 5 % protein solution; 1 : 20, 1 : 100, 1 : 500 and 1 : 2500. Several immune sera were used for each series of the tests, but since similar antisera gave essentially the same results, the reactions in each instance with one typical antiserum only are recorded in the Tables of this paper. The readings, unless otherwise stated, are those obtained after 1 hour.

The extent of precipitin formation is recorded as follows: – (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++ (increasing degrees of precipitation).

Complement-fixation tests.

A range of the antigen solution was made in a series of small test-tubes, each tube containing 0·5 cc. of the diluted antigen; 0·06 cc. of 1 : 5 guinea-pig-serum and 0·10 cc. of the inactivated rabbit-"immune" serum were added to each tube, the tubes shaken and allowed to stand for 45 mins. to 1 hr. 0·5 cc. of a 4 % suspension of sensitised ox-red blood cells was then added to each tube, the tubes shaken and incubated at 37°. The amount of haemolysis after various intervals, usually 15 mins., 30 mins. and 1 hr., was recorded. On some occasions similar series of tests were carried out with 0·05 and 0·15 cc. of immune serum and varying concentrations of antigen. Control tests with no antigen solution, and others with varying amounts of antigen and no immune serum, were always carried out.

The influence of p_H *on the power of horse-serum proteins to react with antibodies to unchanged horse-serum.*

In these experiments samples of normal horse-serum were kept at various hydrogen ion concentrations for 24 hrs. at 19°, and the power of such treated sera to react with antisera to untreated horse-serum was tested at intervals.

The adjustment of the horse-serum to a definite p_H was made by the addition of *N* HCl or *N* NaOH, and to the solution obtained a buffer solution (the universal buffer mixture of Prideaux and Ward [1924] (B.D.H. preparation) of the same p_H was added, the presence of this buffer mixture preventing any appreciable alteration in the p_H of each solution during the course of the experiment. For the solutions to be kept at p_H 1, *N* HCl was added to the diluted serum to give the desired p_H, and the solution then diluted with *N*/10 HCl; *N* and *N*/10 NaOH were used in the same way for the tests at p_H 13.

In the experiment recorded in Table I, 6 cc. of normal horse-serum were diluted with about 20 cc. of 0·9 % NaCl, and *N* HCl or *N* NaOH was added until the desired p_H was obtained; 20 cc. of universal buffer mixture of the corresponding p_H were added, and the mixture diluted to 60 cc. with 0·9 % NaCl. These mixtures were then kept at 19°, and samples withdrawn from each solution at the start, after 45 mins., 3½ hrs. and 24 hrs. Each sample, except those from the solution at p_H 7·5, was neutralised by the addition of acid or alkali and the reaction adjusted to p_H 7·5, after which the required dilutions were made and precipitin tests carried out. In Table I the results with p_H 4, 5, 6, 7, 8, 9 and 10 are omitted, since these did not differ appreciably from those given for p_H 7·5.

Table I. *The influence of* p_H *on the power of horse-serum-proteins to react with antibodies to untreated horse-serum-proteins.*

Tests carried out with anti-horse-serum immune serum No. 107 (readings after 1 hr.).

Duration of treatment at different p_H values	Precipitin reactions with horse-serum-proteins kept for various periods at the following p_H and subsequently neutralised							Dilution* of antigen after neutralisation
	1	2	3	7·5	11	12	13	
Start	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	1 : 20
	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1 : 100
	+	+	+	+	+	+	+	1 : 500
	tr.	tr.	tr.	tr.	tr.	tr.	tr.	1 : 2500
45 mins.	+ ±	+ ±	+ ±	+ ±	+ ±	+	±	1 : 20
	+ ±	+ +	+ +	+ +	+ +	+ +	+	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	f.tr.	f.tr.	tr.	tr.	tr.	tr.	f.tr.	1 : 2500
3½ hrs.	+	+ ±	+ ±	+ ±	+	+	tr.	1 : 20
	+ ±	+ +	+ +	+ +	+ ±	+	±	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	tr.	tr.	tr.	tr.	f.tr.	–	–	1 : 2500
24 hrs.	+	+ ±	+ ±	+ ±	±	±	–	1 : 20
	+ ±	+ +	+ +	+ +	+ +	±	–	1 : 100
	±	±	+	+	+	tr.	–	1 : 500
	f.tr.	f.tr.	f.tr.	tr.	tr.	–	–	1 : 2500

* All antigen dilutions in this and in the following Tables are in terms of dilution of a 5 % protein solution.

The results of these experiments confirm the findings of the earlier workers in that they show marked destruction of antigenicity in alkaline solution and a less pronounced loss by treatment with acid. This investigation of the exact influence of p_H, rather than the study of the action of excess of strong acid

or alkali, leads to the conclusion, however, that in moderately alkaline solutions the loss of the power to give precipitation with antibodies to the unchanged serum is not as rapid as some authors have suggested. Thus, horse-serum kept at p_H 11 for 24 hrs. at 19° shows a slight loss only of this antigenic power. At p_H 12, however, marked loss occurs in $3\frac{1}{2}$ hrs. and at p_H 13 a similar destruction occurs in less than 1 hr.; after 24 hrs. at p_H 13, the serum no longer possesses the power to react with antisera to the native proteins of horse-serum.

In some of the above, and in later experiments, antisera obtained by the injection into rabbits of fat- and lipin-free proteins of normal horse-serum, obtained by the method used by Hartley [1925], were used. In all these experiments, antisera obtained in this way gave results identical with those given by antisera obtained by the injection of untreated horse-serum.

The action of strong alkali on the power of serum-proteins to react with antibodies to the unchanged serum-proteins and the immunological properties of these alkali-treated proteins after iodination and nitration.

Preliminary tests were carried out to determine the amount of NaOH necessary for the destruction, in 20–24 hrs. and at various temperatures (18°, 30° and 37°), of the power of horse-serum-proteins to react with antisera to untreated horse-serum-proteins. After the mixtures had stood for this length of time at the required temperature, conc. HCl was added to give maximum precipitation and the precipitated proteins were centrifuged and dissolved in 0·9 % NaCl solution with addition of the minimum amount of 10 % sodium carbonate solution to give a clear solution. Portions of these solutions of alkali-treated proteins were iodinated or nitrated by the methods indicated above, and the products obtained tested with various antisera. In the majority of these experiments it was found that, although sufficient strong alkali had been added to render the proteins incapable of reacting (in precipitin and complement-fixation tests) with antibodies to untreated horse-serum-proteins, the proteins formed could still take part in immunological reactions after iodination or nitration; thus, after iodination they gave reactions with antisera to iodinated horse-serum-proteins, and after nitration they reacted with antisera to nitrated horse-serum-proteins, the latter observation confirming those of Landsteiner and Barron [1917]. The iodinated alkali-treated proteins were not, however, always as strongly antigenic *in vitro* as were the corresponding nitrated proteins, but usually, after iodination or nitration, these "alkali-proteins" gave good precipitin reactions. In other experiments, where the amount of NaOH was much greater than in the experiments described here, no regeneration of antigenic properties by iodination or nitration could be effected, but this is not surprising in view of the pronounced hydrolytic and other destructive changes which occur when serum-proteins are treated with strong alkali. Table II records an experiment which illustrates some of these features. This experiment, involving the action of NaOH at 37°, shows that

on occasions the antigenic powers *in vitro* can best be restored with HNO_3, for here the nitrated proteins of solution *Y* (a solution of alkali-treated horse-serum-proteins) gave good precipitin reactions with suitable antisera, whereas little precipitation was obtained with iodinated proteins of solution *Y* and antisera to iodinated horse-serum-proteins. In the case of solution *Z*, where much more alkali was used, no appreciable regeneration of the power to give precipitin reactions could be effected by iodination or nitration.

Table II. *Precipitin reactions.*

Antigen	Antigen dilution	Immune serum: Anti-horse-serum (No. 107)	Immune serum: Anti-iodo-horse-serum (No. 1657)	Immune serum: Anti-nitro-horse-serum (No. 1881)
Horse-serum (*X*)	1 : 100	++	–	f.tr.
	1 : 500	+±	tr.	tr.
	1 : 2500	+	tr.	f.tr.
NaOH-treated horse-serum (*Y*)	1 : 100	–	–	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
NaOH-treated horse-serum (*Z*)	1 : 100	–	–	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
Iodinated *X*	1 : 100	tr.	++	tr.
	1 : 500	tr.	±	±
	1 : 2500	–	tr.	tr.
Iodinated *Y*	1 : 100	–	±	–
	1 : 500	–	tr.	–
	1 : 2500	–	–	–
Iodinated *Z*	1 : 100	–	f.tr.	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
Nitrated *X*	1 : 100	–	–	f.tr.
	1 : 500	–	–	+
	1 : 2500	–	–	±
Nitrated *Y*	1 : 100	–	–	+
	1 : 500	–	–	±
	1 : 2500	–	–	f.tr.
Nitrated *Z*	1 : 100	–	–	f.tr.
	1 : 500	–	–	tr.
	1 : 2500	–	–	–

Experimental details.

X. 30 cc. of horse-serum + 1 cc. of toluene.
Y. " " + 6·9 cc. of 5·35 *N* NaOH + 1 cc. of toluene.
Z. " " + 10·5 cc. " + "

These mixtures were kept at 37° for 20 hrs. The "alkali-proteins" of solutions *Y* and *Z* were precipitated by HCl and dissolved as described for the antigens used for injection. The amount of protein in each solution was determined and the antigen dilutions were calculated to refer to 5 % protein solutions.

Table III gives the results of precipitin tests with some of the antigens used for the injection experiments (series (*a*)), and here the restoration of *in vitro* antigenic power to alkali-treated proteins appears to be effected just as readily by iodination as by nitration. Results of a similar nature were obtained in complement fixation tests. The iodinated alkali-treated proteins also reacted with antisera to brominated horse-serum-proteins.

Table III. *Precipitin reactions of the antigens used for the injections (series (a)).*

Antigen	Antigen dilution	Immune serum: Anti-horse-serum (No. 107)	Anti-iodo-horse-serum (No. 1657)	Anti-bromo-horse-serum (No. 1822)	Anti-nitro-horse-serum (No. 1881)
NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	–	–
	1 : 100	–	–	–	–
	1 : 500	–	–	–	–
	1 : 2500	–	–	–	–
Iodinated NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	tr.	–
	1 : 100	–	+ ±	+	tr.
	1 : 500	–	+	±	f.tr.
	1 : 2500	–	f.tr.	f.tr.	–
Nitrated NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	tr.	±
	1 : 100	–	–	tr.	+
	1 : 500	–	–	–	±
	1 : 2500	–	–	–	tr.
Horse-serum	1 : 20	+ +	–	–	–
	1 : 100	+ +	tr.	–	f.tr.
	1 : 500	+	tr.	–	tr.
	1 : 2500	±	f.tr.	–	f.tr.

The in vivo *antigenic properties of alkali-treated proteins and of alkali-treated proteins which have been iodinated or nitrated.*

The results of the experiments described above show that proteins which have been treated with sufficient alkali to destroy the power to react with antibodies to the original protein have not necessarily lost their antigenic capabilities, and that they can function as *in vitro* antigens after iodination or nitration. From these test-tube experiments it would not be justifiable to conclude, however, without further evidence, that restoration of antigenic properties had occurred, for several other possible explanations could be presented. The action of the alkali might, for example, result in the formation of denatured proteins with a new specificity, though such denatured proteins would be expected to give some reaction with antisera to the original protein. The most conclusive evidence would undoubtedly be furnished by immunisation experiments if they showed that, whereas no antibodies are produced in the animal body in response to the alkali-treated proteins, antibodies are readily formed after the injection of the corresponding iodinated or nitrated alkali-treated proteins.

The experiments to be referred to under this heading can be divided into two groups: series (*a*), in which were used horse-serum-proteins which had been treated with sufficient sodium hydroxide solution to give a final concentration approximately *N* and the mixture kept for 22 hrs. at 18°, and series (*b*) with a somewhat similar mixture kept at 30° for 24 hrs. Portions of each solution of alkali-treated proteins were iodinated and others converted into xanthoproteins, and immunisation experiments carried out with the "alkali-protein" and the corresponding iodinated and nitrated proteins. Four rabbits

were used for each antigen solution, and the serum of each animal was tested frequently, in precipitin and complement fixation tests, against the antigen solution used for the injections and against certain other antigens. Each rabbit received seven or eight weekly injections, and the serum tests were made 7 days after the third and subsequent injections. The results of these experiments supported, in general, the conclusion drawn from the results of the experiments already described above, the experiments with the xantho-proteins prepared from the "alkali-proteins" confirming the findings of Landsteiner and Barron [1917]. The antibody response to the iodinated and nitrated compounds, particularly to the former, was perhaps not as marked as was expected, but possibly this can be accounted for by the more drastic treatment with alkali. In these experiments the concentration of sodium hydroxide was approximately the same, but the incubation period was longer than in the experiments of Landsteiner and Barron (22 and 24 hrs. compared with 16 hrs.) and in series (*b*), at least, the incubation temperature was higher. The mixtures prepared by the latter authors were kept at room temperature, whereas those used in the experiments described here were kept at 18° (series (*a*)) and 30° (series (*b*)).

Table IV. *Precipitin reactions between the antigens used for the injections (series (a)) and the sera of the rabbits injected.*

Rabbit No.	Rabbit injected with	Antigen used in test	A Antigen dilution 1:20	1:100	1:500	1:2500	B Antigen dilution 1:20	1:100	1:500	1:2500
120	NaOH-treated serum-proteins (*a*)	NaOH-treated serum-proteins (*a*)	–	tr.	tr.	–	tr.	tr.	f.tr.	–
121			–	–	–	–	–	–	–	–
122			–	tr.	tr.	–	–	tr.	f.tr.	–
123			–	–	–	–	–	–	–	–
124	Iodinated serum-proteins	Iodinated serum-proteins	–	–	–	–	f.tr.	tr.	tr.	–
125	NaOH-treated serum-proteins	NaOH-treated serum-proteins	f.tr.	±	±	f.tr.	tr.	+	±	–
126			–	–	–	–	–	–	–	–
127			–	tr.	f.tr.	–	tr.	±	f.tr.	–
128	Nitrated serum-proteins	Nitrated serum-proteins	tr.	±	±	f.tr.	±	±	tr.	f.tr.
129	NaOH-treated serum-proteins	NaOH-treated serum-proteins	f.tr.	±	±	f.tr.	tr.	±	tr.	–
130			tr.	+	±	f.tr.	+	+	±	f.tr.
131			tr.	tr.	f.tr.	–	f.tr.	tr.	–	–

In the above table, A refers to tests made with sera of the rabbits after these animals had received three injections of the antigen, and under B are given the results with the sera obtained after five injections.

Table IV gives some typical results of the precipitin tests with the sera of the rabbits of series (*a*), and Table V some similar complement-fixation tests. The most pronounced differences are found when the NaOH-treated proteins and the corresponding nitrated compounds are compared. The former engender practically no antibody response, whereas the injection of the latter causes the production of antibodies for the antigen used. The iodinated alkali-treated proteins give some antibody response, and, although it is not as marked as that with the corresponding nitrated compounds, it is not insignificant.

Table V. *Complement-fixation tests with each antigen used for the injections (series (a)) and the sera of the rabbits injected with this antigen.*

Rabbit No.	Proteins used as antigen in test and for immunisation	Antigen dilution used 1:20	1:80	1:320	1:1280	1:2560	Control (NaCl)
120	NaOH-treated serum-proteins	4	4	3	3	4	4
121		4	4	4	4	4	4
122		4	2	1	–	1	4
123		4	4	4	4	4	4
124	Iodinated NaOH-treated serum-proteins	4	4	4	–	1	4
125		4	3	–	–	–	4
126		3	2	1	–	–	4
127		2	1	–	–	–	4
128	Nitrated NaOH-treated serum-proteins	4	2	–	–	–	4
129		2	–	–	–	–	4
30		2	–	–	–	–	4
131		3	2	–	–	1	4

Note. Control tests indicated that the antigen solutions, in the concentrations used here, had neither anticomplementary nor haemolytic powers.

In the above Table, 4 indicates complete haemolysis, – indicates no haemolysis, and 3, 2 and 1 give intermediate degrees of haemolysis.

The results with the proteins of series (*b*) (alkali-treatment at 30°) were similar to those in Tables IV and V, with somewhat less marked precipitin and complement-fixation reactions. Treatment of serum-proteins with strong alkali at this higher temperature probably results in such pronounced hydrolytic and other changes that regeneration, even in part, of antigenic properties is no longer possible. The nitrated proteins of this series, however, did give some antibody response, and marked complement-fixation reactions were obtained. The sera of the rabbits injected with iodinated proteins of this series gave no significant precipitin reactions, but did give some complement-fixation when tested against the corresponding antigen. Thus, in this series, the only satisfactory positive results were obtained in complement-fixation tests.

The sera of all these animals were tested on certain occasions with similar proteins which had not been subjected to the action of strong alkali, *viz.* with horse-serum-proteins, and also with iodinated and nitrated horse-serum-proteins. The results were similar to those described above and recorded in Tables IV and V, with, as was expected, more pronounced precipitation or complement fixation. Thus, the sera of rabbits injected with iodinated alkali-treated proteins reacted with iodinated horse-serum-proteins, but not with nitrated horse-serum-proteins or with untreated horse-serum-proteins, and the sera of rabbits injected with nitrated alkali-treated proteins reacted with the nitrated serum-proteins only.

Discussion.

The destructive action of alkali on protein antigens has long been recognised [Wells, 1909, *etc.*] and several explanations have been put forward to account for this loss of antigenicity. Some loss of this function does occur during similar treatment with acid of approximately the same strength, but this action of acid is very mild compared with that of alkali. Alkalis appear

to have, therefore, a somewhat specific destructive action on protein antigens, and this action does not seem to be related to hydrolytic changes effected in the protein molecule.

The immunological properties of horse-serum-proteins which have been kept in buffered solutions of known p_H for varying lengths of time were first investigated in this work, with results which are of some value in relation to the general study of the immunology of chemically altered protein antigens. As was expected, destruction of the power of such proteins to react with antibodies to the untreated proteins occurred most readily in alkaline solution, but it was rather remarkable that there was no appreciable loss of this function after exposure for 24 hrs. (at 19°) to alkaline solutions at or below p_H 11. At p_H 12 there was moderate, and at p_H 13 marked, loss of this power in less than 1 hr. In acid solutions of p_H 1 and 2 there was only a very slight diminution in 24 hrs. at 19° of the power to give a precipitate with antisera to untreated horse-serum-proteins, and no detectable loss occurred in the solutions kept for this period at p_H 3–10 inclusive. The serum-proteins are certainly not the least sensitive of proteins to treatment with acid or alkali, and thus it appears rather remarkable that such changes in the reaction of the medium can occur without appreciable loss of these immunological properties. Any prolonged exposure of proteins to relatively strongly acid or alkaline solutions will undoubtedly cause serious alteration of the immunological behaviour of these proteins, but from the experiments recorded here it appears justifiable to conclude that exposure for short periods and at the ordinary temperatures to reactions between p_H 2 and p_H 11 will not appreciably alter the protein antigens of serum. In studying the immunological properties of chemically altered protein antigens therefore, although it is always advisable to avoid, where possible, the use of strong acid or alkali, especially the latter, the use of reagents of this type must not necessarily be condemned.

The loss of antigenic properties as a result of the action of alkali might be attributed to any one, or to a combination of two or more, of the changes which occur in the protein molecule. Hydrolysis, racemisation and changes in the sulphur-containing constituents of the protein take place in the alkaline medium, and in all probability there are other ways in which the protein molecule is modified by such treatment. Most authors consider that the loss of antigenicity is related to racemisation. For a discussion of the evidence see Wells [1929] and Hartley [1931]. Hartley suggests, however, that antigenicity is lost before racemisation is complete. No attempt has been made in the work described here to study this relationship or indeed to attempt to relate loss of antigenicity with any definite change or changes in the protein molecule, and the main problem was concerned with the possibility of reversing this destructive action of sodium hydroxide. Regeneration of antigenic power in alkali-treated serum-proteins can be effected by treatment with conc. nitric acid [Landsteiner and Barron, 1917], and the experiments described here have fully confirmed this observation.

The other method used in these attempts to obtain antigenic preparations from non-antigenic alkali-treated serum-proteins involved iodination. Iodination of proteins leads to no loss of antigenic power, but the original species specificity is lost and a new specificity acquired [Obermayer and Pick, 1906], this new specificity being characteristic for the 3 : 5-dihalogenated tyrosine grouping [Wormall, 1930]. Iodination of the alkali-treated proteins used in this work led to some regeneration of antigenic function (*cf.* Tables II, III, IV and V); thus the iodinated alkali-treated proteins reacted *in vitro* with antisera to iodoproteins from horse-serum, and after injection into rabbits they produced characteristic iodoprotein antibodies. The antigenic power regenerated was, however, not very high and certainly was not as pronounced as that released by nitration of the same alkali-treated proteins. It is not to be expected, however, that iodination or nitration will lead to complete regeneration of antigenic power, and the margin between temporary loss (which perhaps is reversed in part by nitration or iodination) and irreversible destruction of antigenicity will probably be small. This loss of antigenicity is probably due to several changes in the protein molecule, and nitric acid (or iodine in ammoniacal solution) may be able to reverse certain changes only. Whatever the reason for the regeneration of antigenic properties, it will probably be concerned with the tyrosine groupings of the protein molecule, groupings which are affected by nitration and iodination, and these findings emphasise still further the significance, from the immunological viewpoint, of the tyrosine molecules which are present in the protein. The species specificity of serum and other proteins appears to be linked in some way with these groupings, although it must not be overlooked that antigenic specificity is not always due to aromatic groupings. The experiments of Landsteiner and Barron and those described here furnish some additional support for the view that the tyrosine molecules play an important part also in determining antigenicity, a view which still rests mainly on the experiments of Wells [1908] and later authors, who have shown that gelatin, a protein deficient in aromatic amino-acids, is non-antigenic.

Summary.

The action of acid and alkali on the power of horse-serum-proteins to react with antibodies to the untreated serum-proteins has been investigated with special reference to the precise influence of solutions of definite p_H values. The results agree with the earlier observations of other authors (Wells, *etc.*) that alkalis have a more marked destructive action than have acids. With moderate degrees of alkalinity, however (up to p_H 11 or so) there is very little loss of these antigenic properties in 24 hrs. at 19°.

In view of the findings of Landsteiner and Barron [1917] that the conversion of non-antigenic alkali-treated-proteins into xanthoproteins results in a regeneration of antigenic properties, attempts have been made to effect the same change by iodination. Some success was obtained, but iodination is not

as efficient as is nitration in regenerating antigenic properties in these alkali-treated proteins. The significance of these findings in relation to the chemistry of protein antigens is discussed.

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CXLIII. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

IV. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF ESTERASES.

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Part III [Stedman and Stedman, 1931] of this series of communications was concerned with an examination of the action towards liver esterase of the group of urethanes which had previously been shown to behave pharmacologically as "parasympathetic stimulants," and it was demonstrated that, while such urethanes possessed the power of inhibiting the action of this enzyme to a very high degree, other urethanes and bases did not produce a similar effect. It was therefore concluded that a relationship between constitution and inhibitory action towards liver esterase existed analogous to that between constitution and physiological action in the same group. This result afforded exceedingly strong, if not conclusive, evidence in favour of Loewi and Navratil's [1926] views regarding the mechanism of the action of physostigmine on the heart, and further appeared to indicate the nature of the enzyme which, according to Engelhart and Loewi [1930] and to Matthes [1930], brings about the destruction of acetylcholine and of the vagus substance. It is clear from Loewi's work, however, that the liver is not normally directly responsible for the destruction of the vagus substance, which, in his original experiments, was shown to be caused by aqueous extracts of the frog's heart. It therefore appeared to be desirable to examine the inhibitory action of the above group of urethanes towards serum esterase, particularly as Engelhart and Loewi, Matthes, and Plattner have shown that both whole blood and serum are capable of destroying acetylcholine. At the same time the actions of the urethanes on two other enzymes, namely pancreatic lipase and phosphatase, the normal substrates of which are esters, have been examined in order to determine the extent to which the inhibitory action of the urethanes is specific.

Pancreatic lipase.

Enzyme preparation. The pancreatic lipase was prepared from pig's pancreas according to Willstätter and Waldschmidt-Leitz's method [1923]. The minced glands were desiccated by successive treatment with acetone and ether and the fibrous material was separated from the dry preparation by pounding it in a mortar and then shaking it through a fine sieve. The fine powder so obtained was extracted for 4 hours at 30° with 87 % glycerol (32 cc. for 2 g. of powder), centrifuged, and the extract stored in this form. Immediately before use, 15 cc. of this extract were diluted to 60 cc. with water, and again centrifuged to remove a fine precipitate which separated.

Hydrolysis of olive oil. The influence of miotine hydrochloride on the hydrolysis of olive oil by pancreatic lipase was first examined. Into each of two small stoppered bottles 2·5 g. of olive oil were weighed and 2 cc. of ammonia-ammonium chloride buffer (p_H 8·9) were then added. The contents of one bottle were now treated with 11 cc. of the above enzyme extract and those of the other with 11 cc. of the same extract containing 5 mg. of miotine hydrochloride which had been dissolved in it one hour previously. Each bottle was shaken for 3 minutes to emulsify the oil and then placed in a thermostat at 30° for one hour. The contents of each bottle were now washed with 100 cc. of rectified spirit into a flask, 20 cc. of ether added to dissolve the oil and the solutions titrated with 0·727*N* alcoholic potassium hydroxide, using thymolphthalein as indicator. In each case 6·8 cc. of the alkali were used. A control, using 11 cc. of water in place of the enzyme solution, required 2·4 cc. of the alkali. It is clear that miotine exerted no inhibitory action.

Hydrolysis of tributyrin. In order to follow the hydrolysis of tributyrin, the stalagmometric method described in Part III was employed. The above diluted extract (1 cc.) of pancreatic lipase was mixed with 2 cc. of phosphate buffer (p_H 7·9) and diluted to 10 cc. with water. One cc. of this preparation was treated with 12 mg. of miotine hydrochloride dissolved in 1 cc. of water and allowed to stand for one hour, when 1 cc. of the mixture was used in a hydrolysis experiment. For the control, 1 cc. of water was employed in place of the solution of miotine hydrochloride. The following figures represent the diminution in the drop number in successive periods of 20 minutes: control, 15, 23; in the presence of miotine, 14, 23. Miotine is thus without inhibitory action on the hydrolysis of tributyrin by pancreatic lipase.

Hydrolysis of methyl butyrate. The method employed was at first identical with that used in Part III with liver esterase, except that the reaction mixture contained a high percentage of glycerol which activates as well as stabilises the lipase. Into a 100 cc. graduated flask were introduced 50 cc. of a 50 % solution of glycerol and 20 cc. of buffer (1 part 2·5 *N* NH_3: 2 parts 2·5 *N* NH_4Cl; p_H 8·9) and the mixture was then warmed to 30° in a thermostat. One cc. of methyl butyrate was dissolved as completely as possible in this by shaking, when 20 cc. of the diluted extract of pancreatic lipase were added and the volume was

made up to 100 cc. with water. The flask was again placed in the thermostat, 20 cc. of the mixture being immediately withdrawn, run into a mixture of 25 cc. of 0·2 *N* hydrochloric acid with 20 cc. of water and titrated with 0·2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar volumes of the mixture were withdrawn at intervals of 20 minutes and titrated in the same way. In the experiment designed to test the inhibitory action of miotine, 12 mg. of the hydrochloride were dissolved in 25 cc. of the diluted extract of the enzyme and allowed to stand for one hour; 20 cc. of this solution were then used in a hydrolysis experiment. The following results were obtained, the figures representing the number of cc. of 0·2 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively: control, 1·75, 2·7, 3·4; in the presence of miotine, 1·15, 1·85, 2·35. A small inhibitory effect is apparent, although it is much smaller in magnitude than with liver esterase.

Willstätter and Memmen [1924] have shown that calcium oleate exerts a marked activating action on the hydrolysis of methyl butyrate by pancreatic lipase. Another experiment was therefore carried out in the presence of this activator. The procedure was identical with that outlined above except that 2 cc. each of 2 % sodium oleate and calcium chloride solutions were added to the reaction mixture after the addition of the enzyme but before making up to volume. In view of the activation caused by this addition, only 5 cc. of the diluted glycerol extract of lipase were used. Nevertheless the same amount of miotine was employed. The following figures are typical of the results obtained: control, 2·25, 4·1, 5·05; in the presence of miotine, 1·7, 3·15, 4·25. Owing to the fact that the hydrolysis of methyl butyrate by pancreatic lipase does not take a linear course, possibly because of the changes which occur in the p_H of the solution, it is not possible to calculate in a simple manner the percentage inhibition produced by the miotine. Nevertheless it is clear from the figures that the inhibition caused by the miotine in this experiment is of the same order of magnitude as that produced by the same quantity in the absence of calcium oleate, although only one fifth of the amount of enzyme was required in the latter experiment.

An experiment similar to that last described was also carried out with the hydrochloride of the methylurethane of *m*-dimethylaminophenol, this particular urethane being chosen because it had proved to be the most active of the urethanes examined in inhibiting the hydrolysis of methyl butyrate by liver esterase. About 10 mg. of the urethane were employed, the remaining details being identical with those described above. The results are shown by the following figures: control, 2·05, 3·9, 4·75; in the presence of the urethane, 1·45, 2·9, 3·85. A small inhibitory effect is again apparent.

In view of the possibility that the glycerol, necessarily present in the above solutions, might diminish the inhibitory action of the urethanes, the influence of glycerol on the inhibitory action of miotine towards liver esterase was examined. An acidified and dialysed extract of liver powder, similar to those

described in Part III, was employed, the hydrolysis of methyl butyrate being followed under the conditions described in that paper with the modification that 50 cc. of water were replaced by 50 cc. of 50 % glycerol. As inhibitor, 0·01 mg. of miotine hydrochloride was employed. The following titration figures were obtained: control (without glycerol), 1·85, 3·8, 5·5; control (with glycerol), 1·85, 3·65, 5·45; in the presence of miotine (without glycerol), 0·55, 1·5, 2·2; in the presence of miotine and glycerol, 0·55, 1·4, 2·0. It is evident that the glycerol was without influence either on the activity of the esterase or on the inhibitory activity of miotine. The experiment further serves to illustrate the much greater sensitivity of liver esterase to miotine. With only a thousandth part of the amount used with pancreatic lipase a greater inhibition was produced.

Kidney phosphatase.

The preparation of kidney phosphatase employed was obtained by Erdtman's method [1927]. 600 g. of pig's kidneys were minced, suspended in 500 cc. of water to which much toluene had been added, and incubated for 2 days at 37°. The mixture was then filtered through a fine metal strainer, and the turbid filtrate treated with 1½ litres of rectified spirit. The precipitate so produced was filtered, stirred with 500 cc. of alcohol and again filtered, this process being repeated twice. It was then similarly treated with 500 cc. of ether, dried in the air and ground in a mortar. Extracts of the phosphatase were prepared by shaking 4 g. of this dry powder with 80 cc. of $N/40$ ammonia for 1½ hours and removing the solid material in the centrifuge.

The substrate employed was sodium glycerophosphate. To a mixture of 10 cc. of 5 % sodium glycerophosphate, 10 cc. of buffer (ammonia-ammonium chloride, p_H 8·9) and 60 cc. of water, previously warmed to 30° in a thermostat, were added 10 cc. of the above solution of phosphatase. The total volume was then made up to 100 cc. with water and the solution replaced in the thermostat. 20 cc. of the mixture were immediately withdrawn and run into 10 cc. of 10 % trichloroacetic acid. The free phosphate was then estimated in 25 cc. of the filtrate by precipitating it as phosphomolybdic acid, dissolving the latter after filtration in 10 cc. of 0·21N NaOH and titrating the excess alkali with 0·0995N nitric acid. Similar withdrawals and estimations were made at fixed intervals. A typical experiment will suffice to illustrate that miotine is without action on kidney phosphatase. 12 mg. of miotine hydrochloride were dissolved in 12 cc. of the enzyme solution and, after an interval of one hour, the phosphatase activity of 10 cc. of the solution was determined, a control being carried out simultaneously. The following figures represent the cc. of nitric acid required to neutralise the excess alkali in estimations on samples withdrawn immediately and after 80 minutes respectively: control, 18·2, 8·9; with miotine, 18·6, 8·9.

Serum esterase.

In order to examine the inhibitory action of the various urethanes towards serum esterase, the serum of the guinea-pig was chosen, since the blood of this

animal is known to contain a relatively high amount of the enzyme. 2–3 cc. of the serum, the exact volume depending upon the esterase activity of the sample employed, were treated with 2 cc. of phosphate buffer of p_H 7·9 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of a solution of the urethane under examination and allowed to stand for about an hour, when the esterase activity of 1 cc. of the mixture was determined by the stalagmometric method described in Part III. In the control experiment, the solution of the urethane was replaced by an equal

Table I. *Inhibition of serum esterase by various urethanes.*

Substrate: tributyrin. T = 20°. p_H = 7·9.

Inhibitor*	Final conc. of inhibitor ($M \times 10^{-3}$)	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Phenyl series:				
Control	—	11	21	—
m-HCl	4	5	11	48
p-HCl	4	8	16	24
o-HCl	4	8	15	29
Control	—	13	25	—
m-MeI	400	5	9	64
p-MeI	400	1	4	84
o-MeI	400	1	3	88
Miotine series:				
Control	—	11	23	—
m-HCl	400	1	2	91
,,	40	5	10	57
p-HCl	400	2	4	83
,,	40	4	10	57
o-HCl	400	1	3	87
,,	40	9	19	17
Control	—	14	27	—
m-MeI	4000	2	3	89
,,	400	11	22	19
p-MeI	4000	1	3	89
,,	400	13	23	15
o-MeI	4000	1	2	93
,,	400	9	17	37
Benzyl series:				
Control	—	12	23	—
m-HCl	40	2	5	78
,,	4	8	16	30
p-HCl	40	3	7	70
,,	4	9	18	22
o-HCl	40	6	12	48
Control	—	14	26	—
m-MeI	400	8	17	35
p-MeI	400	6	14	46
o-MeI	400	7	15	42

* *Phenyl series*; methylurethanes of the isomeric dimethylaminophenols.
Miotine series; methylurethanes of the isomeric α-hydroxyphenylethyldimethylamines.
Benzyl series; methylurethanes of the isomeric hydroxybenzyldimethylamines.

volume of water. The results obtained are given in Table I. The urethanes employed were identical with those used in Part III and are indicated by the same abbreviated names.

Liver esterase.

In view of the fact that serum from the guinea-pig was used in the above experiments it was thought that it would be of interest to examine the inhibitory actions of the various urethanes on the liver esterase from the same species. A preparation of this esterase was therefore made from a number of guinea-pig's livers using the same procedure as was employed in Part III in connection with liver esterase from the pig. The activity of the preparation towards methyl butyrate, however, proved to be much smaller than that from the pig and it was not, therefore, possible to follow the hydrolysis of simple esters by the technique employed in Part III. Despite its smaller activity towards methyl butyrate, this preparation nevertheless proved to be at least as active towards tributyrin as was the esterase from pig's liver. The experiments recorded in Table II were therefore carried out, using this substance as

Table II. *Inhibition of guinea-pig's liver esterase by various urethanes.*

Substrate: tributyrin. T = 20°. p_H = 8·0.

Inhibitor	Final conc. of inhibitor ($M \times 10^{-3}$)	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Phenyl series:				
Control	—	14	26	—
m-HCl	40	0	0	100
p-HCl	40	9	18	31
o-HCl	40	7	16	38
Control	—	14	27	—
m-MeI	400	6	13	52
p-MeI	400	3	8	70
o-MeI	400	7	16	41
Miotine series:				
Control	—	11	22	—
m-HCl	40	4	8	64
p-HCl	40	6	14	36
o-HCl	40	4	8	64
Control	—	13	24·5	—
m-MeI	400	5	10·5	57
p-MeI	400	5	11	55
o-MeI	400	5	10·5	57
Benzyl series:				
Control	—	13	24	—
m-HCl	40	3	8	67
p-HCl	40	7	16	33
o-HCl	40	4	9	63
Control	—	14	27	—
m-MeI	400	4	9	67
p-MeI	400	5	12	56
o-MeI	400	6	13	52

substrate. The preparations of the esterase employed were obtained by extracting the desiccated liver powder with dilute ammonia, acidifying this extract with acid and, after removing the precipitate thus produced, dialysing for about 3 days in collodion membranes. In the various experiments recorded,

1·5–3 cc. of such an extract were mixed with 2 cc. of phosphate buffer of p_H 8·0 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of water or of a solution of the urethane under examination, allowed to stand for about an hour, and 1 cc. of the mixture used in a hydrolysis experiment. The results obtained with the various urethanes are recorded in Table II.

Summary and discussion.

In so far as they show that the hydrolysis of tributyrin by the esterase from the liver of the guinea-pig is inhibited by small concentrations of the urethanes which have been shown to behave as parasympathetic stimulants, the above experiments constitute an extension to the liver esterase from a second species of the results obtained with the pig and reported in Part III of this series. While we have not carried out any extensive experiments with liver esterases from other species, we have nevertheless submitted those from a few to a preliminary examination and have found in each case that their activity is inhibited by miotine when present in concentrations of the same order of magnitude as employed in the above experiments. It would therefore appear legitimate to conclude that the urethanes of the type under consideration inhibit the activity of liver esterases in general and irrespective of the species from which they are derived.

Our experiments further demonstrate that the inhibitory action of the urethanes is not restricted to the liver enzyme but extends to the esterase which is present in the blood-serum of the guinea-pig and of certain other species. In view of the fact that it has been shown by the authors mentioned in the introduction that both whole blood and serum are capable of destroying small amounts of acetylcholine, a process which is inhibited by physostigmine, this result was, perhaps, to be expected. Nevertheless, it must be emphasised that the experiments here recorded have been carried out using tributyrin as substrate. While it would seem probable that the same enzyme is concerned in both processes, this cannot at present be regarded as definitely established. There exists, indeed, some evidence to the contrary. Thus, Takahashi [1930], has recently compared the lipolytic activities of the sera from a number of species, using tributyrin as substrate, and has found that they fall in the following order: rabbit > cat > horse > man > dog > cattle. This order is in general agreement with the results of earlier workers and also with some unpublished experiments which we have carried out. On the other hand, the order in which the defibrinated blood from a number of species destroys acetylcholine is, according to Galehr and Plattner [1927], man > pig $\geqq$ cattle > dog > horse > rabbit > cat. The latter series is practically the reverse of that which holds for the hydrolysis of tributyrin and hence suggests either that different enzymes are concerned in the two processes or that some other factor, hitherto unrecognised, is involved. It is unlikely that a solution of this aspect of the problem will be possible until the blood-enzymes have been purified and

concentrated. Unfortunately such purification is attended with considerable difficulty on account of the quantity and ready solubility of the serum proteins. We are nevertheless at present engaged in an attempt to effect this purification.

Although the possibility thus exists that the hydrolysis of tributyrin and the destruction of acetylcholine are brought about by different serum enzymes, our results appear quite definitely to indicate that the enzymes, if specifically different, are of the same general nature. We have now established that the activities of both liver and serum esterases are inhibited by small concentrations of urethanes of the miotine type. Nevertheless, the above results show that the activity of pancreatic lipase, an enzyme which, although it resembles liver esterase in hydrolysing simple esters, differs from the latter enzyme in attacking fats relatively more rapidly, is not inhibited by miotine when its substrate is either a fat, in the form of olive oil, or tributyrin. When, however, its substrate is methyl butyrate, a simple ester, its action is inhibited by miotine, although a concentration of the drug which is high compared with that required with serum or liver esterase is necessary. Similarly, the hydrolysis of glycerophosphoric acid by kidney phosphatase, a process involving an ester of a different type from that attacked by liver esterase, is unaffected by miotine. It thus appears that the inhibitory action of the urethanes with which we are concerned is directed mainly towards true esterases, and it is therefore probable that the destruction of acetylcholine is brought about by an enzyme of the same nature.

Regarding the nature of the inhibitory action of the drugs in question, there appears little to be added to our comments in Part III. It was pointed out in that communication that the urethanes are esters and that combination between them and the enzyme probably involves the same mechanism as with the normal substrate, in which case the resulting inhibition would be due to the inability of the enzyme to hydrolyse the urethane. Some chemical evidence which supports this view may be mentioned. In Part I [1926] of this series it was mentioned that when aqueous solutions of certain of the urethanes are boiled, decomposition occurs with the production of methyl *iso*cyanate. This decomposition was erroneously referred to as hydrolysis, but it is clear, as pointed out by Aeschlimann and Reinert [1931] in a paper concerned with the pharmacology of urethanes of the same type, that no hydrolytic process is involved. This can be seen from the following equation:

$$N(CH_3)_2.CH(CH_3).C_6H_4.O.CO.NHCH_3 = N(CH_3)_2.CH(CH_3).C_6H_4.OH + CH_3CNO.$$

If, then, despite their structures as esters, these urethanes are not normally hydrolysed by ordinary chemical reagents but undergo a decomposition of a different nature, it is not difficult to understand the inability of esterases to bring about their destruction. The inhibitory action would be a direct consequence of this, provided the urethanes were endowed with high affinities for the enzyme.

The general relations between the nature of the urethane and its inhibitory activity towards liver esterase observed in Part III and discussed in that paper have again been encountered in the experiments described above and require no further comment. The order of activity of isomeric urethanes appears, however, to differ according to the source of the enzyme. While it would be in accordance with other observations that the enzymes from different species should exhibit differences in this respect, it is more difficult to understand why the esterases from the liver and serum of the same species should show similar differences. The obvious explanation, that the two latter enzymes are different substances, may be correct. We feel, however, that it would be wiser at the present time not to lay too much stress upon these small differences, which might conceivably be caused by impurities which are present in the enzyme solutions.

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CXLIV. NOTE ON THE INTERNATIONAL STANDARD FOR THE ANTINEURITIC VITAMIN B_1.

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The vitamin B_1 preparation adopted as International Standard by the League of Nations Conference on Vitamin Standards, held in London in June 1931, is an adsorbate on fuller's earth obtained by Seidell's method [1916] from an extract of rice polishings, the extract being made with water containing 20 % alcohol and acidified with sulphuric acid to p_H 4·5 [League of Nations Health Organisation, 1931]. 3 kg. of the fuller's earth represent the antineuritic vitamin B_1 from 100 kg. of rice polishings. This adsorbate, which is prepared in the Medical Laboratory at Batavia (Java) and used in that district for the treatment of beriberi, formed the starting-point for the preparation of the crystalline vitamin B_1 product isolated by Jansen and Donath [1927].

Before its adoption as a vitamin B_1 standard, this "acid clay" adsorption product was investigated by several members of the Conference to ascertain its degree of suitability for use as a standard. At the request of the Accessory Food Factors Committee (of the Medical Research Council and Lister Institute) quantitative tests using different methods were made in various laboratories in this country and abroad; in these tests the adsorbate was used as a standard of comparison in the estimation of the potency of other vitamin B_1-rich substances—dried yeast, wheat embryo, *etc.* Standard specimens of these materials were distributed to the various laboratories and in the course of tests carried out at the Lister Institute the following results were obtained which seem worthy of record, as being possibly of general interest and of use to other workers in this field.

In the method of vitamin B_1 estimation employed in the following tests, the criterion adopted was the growth of young rats [Chick and Roscoe, 1929]. A basal diet was used (caseinogen 100; rice starch 300; salt mixture McCollum No. 185, 25; arachis oil 75; water 550; with cod-liver oil 50–100 mg. daily administered separately), to which the antidermatitis vitamin B_2 was added as a small daily ration of an autoclaved (4 hours, 120°, at p_H 5·0) watery yeast extract given in amount equivalent to 0·5 g. of the yeast (dry wt.) from which the extract was prepared [see Roscoe, 1930]. The standard degree of "normal" growth adopted was an average weekly increase in body weight of 10–14 g. While the tests were in progress there was an exceptionally cold period, lasting about two weeks, during which it was impossible to keep the animal room at the usual temperature and the growth of the rats under observation was checked. In calculating the average weekly increments in growth, those corresponding to this period have been omitted.

The vitamin B_1 standard adsorption product is a fine white powder easy to administer and readily taken. The minimal daily dose required to support an average weekly increase of 10–14 g. was found to be about 10 mg. (see Table I) and for convenience in preparing the doses the material was diluted ten-fold with pure dextrin and thus administered. The results of the series of tests summarised in Table I show the standard to be about 6 and 12 times as

Table I. *Comparative (antineuritic) vitamin B_1 value of* (a) *the standard adsorption product from rice polishings,* (b) *dried yeast,* (c) *wheat embryo, and* (d) *Peters's antineuritic concentrate from yeast.*

(Vitamin B_2 supplied as autoclaved yeast extract, daily dose≡0·5 g. yeast (dry wt.).)

Material	Daily dose g.	No. of rats used	Av. weekly increment in weight during 5 weeks' observation g.	Av. weekly increment in growth during 1st week g.	Av. weekly increment in growth during 5th week g.
(*a*) International Standard ...	0·025	1	16·2		
	0·012	6	14·3	15·0	13·5
	0·009	4	11·5	15·0	8·2
	0·006	4	7·7	8·5	6·5
International Standard after 10–12 months in refrigerator	0·010	2	11·4	19·0	9·0
	0·0075	2	9·7	13·0	4·0
International Standard after 10–12 months at 37°	0·010	2	10·6	14·5	11·0
	0·0075	2	8·1	14·0	5·0
(*b*) Dried yeast	0·10	3	17·5	17·5	12·0
	0·075	5	14·6	17·2	11·0
	0·05	5	10·9	9·7	11·5
(*c*) Wheat embryo ...	0·20	4	16·4	17·0	10·0
	0·15	4	15·2	17·7	9·2
	0·10	6	10·3	13·5	7·2
	cc.				
(*d*) Peters's antineuritic concentrate from yeast (0·1 cc.≡0·6 g. yeast dry weight)	0·10	4	17·2	18·0	17·0
	0·075	2	17·4	21·0	15·0
	0·05	3	15·4	17·7	11·0

potent respectively as the samples of dry yeast and wheat embryo tested. The International unit of vitamin B_1 is defined as the "antineuritic activity of 10 milligrams of the international standard adsorption product" [Report, 1931, p. 76]. The sample of dried yeast examined would therefore contain 16–18 International vitamin B_1 units per g. and the sample of wheat embryo 8 units per g.

It has frequently been found that when young rats are fed on a simplified "complete" diet containing the minimum amounts of B-vitamins for "normal" growth, the rate of increase in weight may show a falling off after a few weeks; in the estimation of vitamin B_1 this may happen towards the end of the five weeks' period of the test (see Table I, column 5). In the present experiment this occurred to some extent when vitamin B_1 was given as dried yeast, but to a greater extent when wheat embryo or the standard adsorption material from rice polishings was used and more especially after the latter material had been stored for a year (Table I).

It is noteworthy that in most investigations of the B-vitamins, those factors have been studied as they occur in yeast and yeast products. Failure in continued growth on a "complete" experimental diet has frequently been observed when the yeast products have been purified or when the constituents of the B-vitamin complex have been derived from other natural substances. To explain the relative failure when vitamin B_2 was supplied by a preparation from egg-white rather than by an autoclaved yeast extract Chick and Copping [1930] and Roscoe [1930] postulated the existence of factor Y, an unknown constituent of the yeast vitamin complex abundantly present in yeast and some other foods and lacking in egg-white. In the present tests, however, factor Y would be provided in the autoclaved yeast extract given as source of vitamin B_2. The relatively heat-labile vitamin B_4, found by Reader [1929, 1930, 1, 2] to be necessary for rat growth, would be supplied in the acid clay ration, seeing that the crystalline vitamin B_1 product finally obtained from this material by Jansen and Donath's method was found by Jansen *et al.* [1930] to be an unusually rich source of vitamin B_4.

It is probable that our knowledge of the B-vitamin complex might be extended and some of the discrepancies characteristic of the subject removed by a further study of these vitamins as they occur in natural materials other than yeast. In the meantime the existing evidence suggests the presence in yeast of other essential dietary factors in addition to those of the vitamin B complex which have been already recognised.

Stability of the standard vitamin B_1 adsorption product. This material was reported from the Medical Laboratory, Batavia, to possess great stability. In the present investigation tests made after storage for 10–12 months, both in a refrigerator and in an incubator room maintained at 37°, showed little, if any, deterioration to have taken place in the original vitamin B_1 potency (Table I).

Content of vitamin B_2. A few growth tests were made on young rats receiving diets in which the standard adsorption product was given as source of vitamin B_2, and vitamin B_1 was provided as Peters's purified concentrate from yeast (method of Chick and Roscoe [1928]). Only an insignificant degree of growth (3–4 g. weekly) was obtained with daily doses of 0·05 and 0·1 g.; with 0·25 g. daily the average weekly increment in weight was only 6·5 g. The vitamin B_1 content of the standard material is therefore more than 25 times, and probably at least 50 times, greater than its content of vitamin B_2 and the amount of the latter contained in the small daily dose of 10 mg. required to supply vitamin B_1 might be regarded as negligible.

This standard vitamin B_1 could therefore be used as a source of vitamin B_1, sufficiently free from vitamin B_2, in diets destined for work on the latter vitamin and would provide a much more convenient and economical material for the purpose than Peters's vitamin B_1 concentrate from yeast, which is only obtained after an elaborate and lengthy process of preparation.

SUMMARY.

1. The preparation adopted as International Standard for the antineuritic vitamin B_1 (adsorption product on fuller's earth from an acid, watery extract of rice polishings) has been found exceedingly potent (daily rat dose about 10 mg.), convenient to handle and easy to administer.

2. It possesses a satisfactory degree of stability when preserved for a year either at room temperature, at 37° or in a refrigerator.

3. Its content of vitamin B_2 was found to be negligible in comparison with its vitamin B_1 potency. This material should therefore serve as a useful source of vitamin B_1 in diets designed for the study of vitamin B_2.

In conclusion our best thanks are due to Prof. B. C. P. Jansen for the supply of the standard vitamin B_1 adsorption product with which the above tests were made.

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CXLV. EQUOL, A NEW INACTIVE PHENOL ISOLATED FROM THE KETOHYDROXY-OESTRIN FRACTION OF MARES' URINE.

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The discovery by Zondek [1930] that the urine of pregnant mares contained large amounts of the oestrus-producing hormone has resulted in the widespread adoption of this material as the starting-point for the large scale manufacture of the hormone. It appears that the isolation of the crystalline hormone from mares' urine in a chemically pure condition is a somewhat more difficult problem than its isolation from human urine. De Jongh, Kober and Laqueur [1931], Nieuwenkamp and Kober [1931], and Dingemanse *et al.* [1931], working in Laqueur's laboratory, have reported that the crystalline active substance present in mares' urine is identical with their crystalline "menformon" (ketohydroxyoestrin) isolated from human urine. These workers found that the crude crystallisates were contaminated with other substances possessing considerably lower melting-points. By frequent crystallisations they were able however to separate the pure hormone.

More recently Girard *et al.* [1932] have shown that the hydroxyketone fraction of mares' urine concentrates contains, in addition to ketohydroxyoestrin, two other active crystalline substances which appear to have one more double bond than the former and which differ in their optical rotations.

It is clear therefore that processes, which when applied to human urine extracts yield pure ketohydroxyoestrin, may fail to do so when applied to mares' urine owing to the presence of other crystalline substances having similar solubilities and similar chemical properties.

Recently the authors have had the opportunity of examining a large quantity of crude mares' urine extract which was put at their disposal by Schering-Kahlbaum A.G., Berlin.

The total phenolic fraction was separated by the method previously described [Marrian and Haslewood, 1932], and this was then fractionated between benzene and aqueous alcohol. The benzene-soluble fraction containing the ketohydroxyoestrin fraction [*cf.* Butenandt, 1930] was then distilled in a

high vacuum. This process, when previously applied to human urine extracts, invariably yielded a crystalline or semi-crystalline sublimate from which it was easy to isolate pure ketohydroxyoestrin by washing with cold ether and crystallising from aqueous alcohol. In the case of the mares' urine extract, the product was found to be a clear, pale yellow glass. This material was all easily soluble in cold ether and no crystalline substance with a melting-point within 10° of ketohydroxyoestrin could be separated by direct crystallisation. The ketohydroxyoestrin was therefore separated by solution in the minimum of dilute alkali and ether-extraction [*cf.* Veler, Thayer and Doisy, 1930].

From the aqueous alkaline solution, on saturation with carbon dioxide, large amounts of a buff-coloured amorphous solid were precipitated. This substance crystallised readily from benzene or aqueous alcohol in nearly colourless needles which melted after several recrystallisations at 189°–190·5°. On analysis it was found to have the formula $C_{15}H_{14}O_3$. It exhibited no oestrus-producing action when injected into ovariectomised mice in doses as large as 0·086 mg. (1 m.u. of trihydroxyoestrin = 0·000125 mg.).

On methylation with methyl sulphate and sodium hydroxide a compound was formed which analysis proved to be a dimethyl ether. It was clear therefore that two oxygen atoms were present in phenolic hydroxyl groups.

Acetylation and benzoylation yielded a diacetate and a dibenzoate respectively, which suggested that the third oxygen atom was not present in a non-acidic hydroxyl group.

Both the phenol itself and its dimethyl ether were methylated, acetylated and benzoylated under various conditions, but in no case was any indication obtained of a third reactive hydroxyl group. Since it was possible that a third hydroxyl group might be present which was resistant to esterification, an experiment was carried out in which the dimethyl ether was treated with Grignard's reagent (CH_3MgI) in amyl ether. No evolution of methane occurred. We are of the opinion that these experiments definitely exclude the possibility of a third hydroxyl group.

Neither the phenol itself nor its dimethyl ether could be induced to react with hydroxylamine or 2 : 4-dinitrophenylhydrazine which suggested that the third oxygen atom was not ketonic, although the possibility of a non-reactive ketonic group could not be excluded.

The evidence, although of a negative character, suggests that the third oxygen atom may be present in an ether linkage. Determination of the methoxyl content of the dimethyl ether, however, proved the absence of methoxyl groups or other ether groups in the phenol itself which would readily yield volatile iodides on hydrolysis with hydriodic acid. The authors are inclined to favour the view that the third oxygen atom is present as a cyclic ether.

In view of the source of this new phenolic compound, the name "equol" is suggested as being suitable.

Experimental.

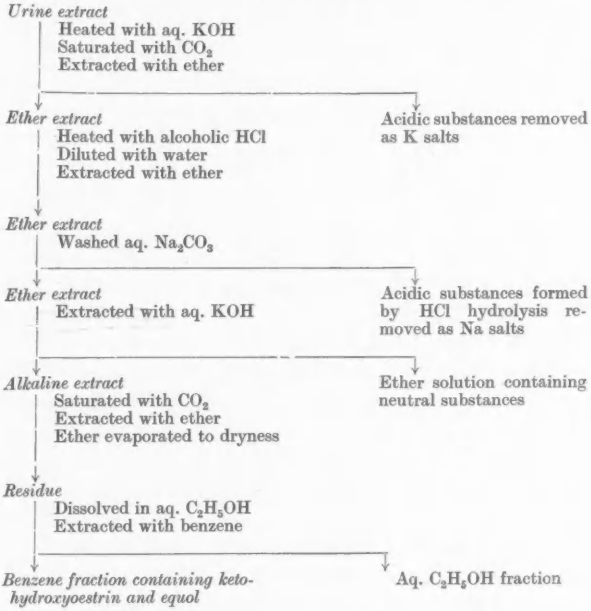
Methods. Melting-points were determined with short-stemmed Anschütz thermometers. No corrections were applied.

Optical rotations were observed in a 0·5 dm. Fischer micro-tube, using the mercury green line.

Micro-determinations of carbon, hydrogen, halogens and methoxyl groups were carried out by Dr Ing A. Schoeller, Berlin. Micro-molecular weight determinations were made by Rast's method. All substances before analysis were dried over phosphorus pentoxide in high vacuum.

Isolation of equol. The scheme adopted for the separation of the keto-hydroxyoestrin fraction of the mares' urine extract is shown in Table I.

Table I.



The benzene solution containing the ketohydroxyoestrin and equol was evaporated to dryness and distilled for 12 hours at 100–110°/0·01–0·03 mm. Hg. This distillate was discarded. The temperature was then raised to 160–180° for several days. The glass-like sublimate was collected, and dissolved in the minimum amount of hot dilute aqueous sodium hydroxide, diluted heavily with water and extracted repeatedly with ether to remove the ketohydroxy-oestrin. The aqueous alkaline fraction was then saturated with carbon dioxide and the crude equol, which was precipitated, filtered off, washed thoroughly

with water and dried. The yield from 1 kg. of crude urine extract, representing about 500 litres of urine, was about 3 g.

Purification and properties of equol. The crude equol after decolorising with charcoal in alcoholic solution was crystallised from aqueous alcohol and from benzene. A sample crystallised once from aqueous alcohol and four times from benzene melted at 189–190·5°.

Equol was readily soluble in ethyl and methyl alcohols, ether, ethyl acetate and acetone. In cold benzene, cold water, chloroform and light petroleum, the solubilities were low. In view of these solubilities, it is remarkable that equol appears in the benzene fraction rather than the aqueous alcoholic fraction in the fractionation of the total urine-phenols. The fact was confirmed however by a separate experiment on a pure sample of equol.

Equol gave strong Millon and xanthoproteic reactions. No colour was given with ferric chloride either in dilute aqueous solution or in aqueous alcoholic solution.

Optical rotation of equol. Rotations were determined in alcoholic solution on several different samples of equol of different degrees of purity. The results are shown in Table II.

Table II. *Optical rotation of equol.*

Times recryst.	M.P. °C.	Wt. taken in mg.	Alcohol cc.	Temp.	α	$[\alpha]_{5461}$	Batch No.
2	184–187	3·15	0·40	20°	−0·086°	−21·8°	1
2	184–187	11·06	0·50	19°	−0·236°	−21·3°	1
2	184–187	3·20	0·54	20°	−0·056°	−18·8°	1
1	187–189	9·35	0·50	19°	−0·170°	−18·2°	2
1	187–189	9·92	0·50	19°	−0·226°	−22·8°	2
5	189–190·5	9·68	0·50	20·5°	−0·212°	−21·9°	2*a*
5	189–190·5	7·95	0·50	21°	−0·202°	−25·4°	2*a*
					Mean	−21·5°	

Although there is not perfect agreement between the figures obtained by different determinations on the same batch, the results are as satisfactory as can be expected working with such small amounts of material. It is clear that the rotation does not vary appreciably with the extent of purification and accordingly it is concluded that it is a definite property of equol itself rather than due to a small amount of optically active impurity.

Analysis of equol. Sample M.P. 189–190·5°.

4·470 mg. gave 12·145 mg. CO_2, 2·360 mg. H_2O;
4·310 mg. gave 11·695 mg. CO_2, 2·290 mg. H_2O;
0·23 mg. in 2·70 mg. camphor, Δ, 15·0°, 14·6°;
0·21 mg. in 2·04 mg. camphor, Δ, 16·0°, 16·0°.

(Found: C, 74·11, 74·02; H, 5·91, 5·94 %. Mol. wt., 230, 236, 255. $C_{15}H_{14}O_3$ requires: C, 74·38; H, 5·78 %. Mol. wt., 242.)

Equol dimethyl ether. 80 mg. of equol (M.P. 184–187°) were dissolved in about 10 cc. of *N*/2 sodium hydroxide and shaken for several hours with 2 cc. of dimethyl sulphate. The solid which separated was filtered off, washed

thoroughly with water, dried and crystallised from absolute ethyl alcohol. 45 mg. of the crystallised product, M.P. 88·5–89·5°, were obtained. The product was insoluble in aqueous alkali and gave no Millon reaction.

4·550 mg. gave 12·205 mg. CO_2, 2·630 mg. H_2O;
5·300 mg. gave 14·415 mg. CO_2, 3·130 mg. H_2O;
3·802 mg. gave 6·455 mg. AgI (micro-Zeisel).
0·24 mg. in 2·58 mg. camphor, Δ, 14·5°, 15·0°;
0·22 mg. in 3·32 mg. camphor, Δ, 9·5°.

(Found: C, 75·86, 75·56; H, 6·70, 6·73 %; CH_3O, 22·59 %. Mol. wt., 251, 245, 244. $C_{15}H_{12}O.(OCH_3)_2$ requires: C, 75·55; H, 6·66; CH_3O, 22·96 %. Mol. wt., 270.)

Equol diacetate. 53·9 mg. of equol (M.P. 184–187°) were heated with 3 cc. acetic anhydride for 3 hours at 100°. After cooling, excess of water was added and the precipitated acetate washed and dried. The crude product was recrystallised from absolute methyl alcohol. 25 mg. of the recrystallised acetate, M.P. 122·5°, were obtained.

4·281 mg. gave 10·900 mg. CO_2, 2·130 mg. H_2O;
0·23 mg. in 1·82 mg. camphor, 1st M.P. Δ, 20°.

(Found: C, 70·08; H, 5·58 %. Mol. wt., 316. $C_{15}H_{12}O.(OOC.CH_3)_2$ requires: C, 69·94; H, 5·52 %. Mol. wt., 326.)

Equol dibenzoate. 63·8 mg. of equol (M.P. 164–167°) were heated for a few minutes with 3 cc. benzoyl chloride. The mixture was cooled and then shaken for several hours with an excess of 2*N* NaOH. The crude benzoate which separated was filtered off, washed thoroughly and dried. The product was recrystallised twice from a mixture of equal parts of chloroform and absolute methyl alcohol. About 80 mg. of recrystallised benzoate were obtained. The dibenzoate showed a clear double melting-point. At 187–189° it melted to a translucent liquid; at 223·5–225°, the melt cleared sharply. Just below 223° a vivid play of colours was observed in the melt. Clear evidence of liquid crystal formation was gained by examination of the melt by polarised light.

4·719 mg. gave 13·080 mg. CO_2, 2·080 mg. H_2O;
0·29 mg. in 2·62 mg. camphor, 1st M.P., Δ, 10·0°;
0·186 mg. in 1·68 mg. camphor, 1st M.P., Δ, 10·5°.

(Found: C, 76·83; H, 4·98 %. Mol. wt., 445, 438. $C_{15}H_{12}O.(OOC.C_6H_5)_2$ requires: C, 77·33; H, 4·89 %. Mol. wt., 450.)

Summary.

A dihydroxyphenol, $C_{15}H_{12}O(OH)_2$, M.P. 189–190·5°, $[\alpha]_{5461} = -21·5°$, has been isolated from the ketohydroxyoestrin fraction of pregnant mares' urine. This substance has been designated equol.

Equol has no oestrus-producing action.

The following derivatives of equol have been prepared: dimethyl ether, $C_{15}H_{12}O(OCH_3)_2$, M.P. 89°; diacetate, $C_{15}H_{12}O(OOCCH_3)_2$, M.P. 122·5°; dibenzoate, $C_{15}H_{12}O(OOCC_6H_5)_2$, M.P. 187–189° and 223·5–225° (liquid crystal formation).

The authors wish to express their sincere thanks to Prof. W. Schoeller of Schering-Kahlbaum A.G., Berlin, for supplying the extract used in this work and for giving them information concerning its preparation.

They are again deeply indebted to the Medical Research Council for a grant to one of them (G. F. M.), out of which the expenses of the research were defrayed.

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CXLVI. SURFACE FILMS OF OESTRIN DERIVATIVES.

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Introduction.

This paper describes the results of surface pressure and surface potential measurements on various derivatives of oestrin, spread in the form of unimolecular films on water surfaces. The work was undertaken in the hope of throwing light on the constitution of the molecules, as well as for the intrinsic interest attaching to the surface properties of substances of such biochemical importance as the oestrous hormone.

The recent work of Butenandt, Marrian, Doisy, and their collaborators [*cf.* Marrian, 1932] has shown that at least two oestrus-producing substances are present in the urine of pregnant women, namely (*a*) trihydroxyoestrin $C_{18}H_{21}(OH)_3$ and (*b*) ketohydroxyoestrin $C_{17}H_{21}CO(OH)$. The former compound contains two hydroxyl groups one of which is phenolic; the latter one phenolic hydroxyl and one ketonic group. The fact that trihydroxyoestrin may be converted into ketohydroxyoestrin by distillation with sodium bisulphate clearly shows the close chemical relation between the two compounds and suggests that the two alcoholic hydroxyl groups in the former compound, from which water is eliminated to form the hydroxyketone, are attached to adjacent carbon atoms.

Little so far is known with certainty concerning the constitution of these compounds. It is evident that at least one benzene nucleus is present, while the work of Doisy and his colleagues [Thayer, Levin and Doisy, 1931, 1, 2] suggests the presence of one double bond which reacts readily with halogens. Butenandt and co-workers [1932] have recently discussed the question of the presence or absence of a reactive double bond. They suggest tentatively that an "enolic" double bond is present in the molecule in addition to the three double linkages in the benzene ring.

If it is assumed that a double bond is present, then it is probable that the carbon skeleton is a substituted partially reduced three benzene ring system.

Such a compound might belong to the group of (*a*) diphenylbenzene, (*b*) phenanthrene or anthracene, (*c*) phenylnaphthalene. If the double bond were not present these structures would still be possible if a bridged ring were present.

With the assumption that no double bond or bridged ring is present a condensed four benzene ring structure seems most probable. Another possibility is a structure based on the Windaus-Wieland formula for sterols. The well known facts that, during pregnancy, there is some disturbance of sterol metabolism, and that the sterol-like compound, pregnandiol, is present together with the hormone in the urine of pregnant women, might suggest a connection between oestrin and the sterols. On the basis of Rosenheim and King's [1932] recently suggested chrysene structure for sterols, the condensed four ring structures are again supported. In this connection, however, if the chrysene suggestion proves to be incorrect, other four ringed structures containing one or more five membered rings are possible. Certain of these skeletons appear less probable on consideration of the chemical evidence. The diphenylbenzenes and phenylnaphthalenes ought easily to yield aromatic acids of lower molecular weight on fusion with potash, but in numerous experiments by two of us (G. A. D. H. and G. F. M.) no indication of such products has been found in brief fusions. The main product of potash fusion has been found to be (unpublished experiments by the above authors) an eighteen carbon atom dicarboxylic acid. Since this is extremely stable to potash fusion it is more probable that a condensed ring structure is present. It will be seen that the surface film results are in accordance with this view, as also are the crystallographic measurements of which Bernal [1932] has given a preliminary account.

Butenandt and his collaborators [1932] have come to the conclusion that a phenanthrene structure is probable. While the findings in this paper provide support for this view, the possibility of four ring structures is equally probable.

Experimental.

The surface pressure (the outward force on a barrier in the surface dividing the film-covered surface from a clean water surface) was measured by the apparatus of Adam and Jessop [1926, 1] and Adam [1930, p. 37]. The substances were weighed out in small stoppered bottles, in quantities of 1–2 mg.; sufficient alcohol was added to dissolve the substance, and the solution was diluted to about 2 cc. with benzene. This solution was dropped on to the surface from a calibrated dropping pipette, employing the corrections for evaporation of the solvent described by Adam and Jessop [1926, 2]. The error in the measurement of the amount put on the surface is estimated as of the order of 3 %.

The films were examined for collapse by the method of Zocher and Stiebel [1930], in which a cardioid condenser is inserted through the bottom of the trough, giving intense dark-ground illumination of the surface. Collapsed aggregates show up in this way as bright spots on a dark ground, whereas perfect unimolecular films show nothing.

The change in the electrical potential at the air-liquid surface was measured by a modification of the method recently published by Schulman and Rideal [1931] an account of which will be published shortly. We are greatly indebted to Mr J. B. Harding for assistance in making the surface potential measurements.

Experimental results.

Trihydroxyoestrin, and ketohydroxyoestrin, also the acetyl derivative and the oxime of the latter, dissolved too rapidly in the water for any measurements to be made on the films. The methyl ether of the ketone and the trimethyl ether of trihydroxyoestrin formed unstable, rapidly collapsing films, on which no measurements could be made. The triacetyl derivative and the diacetyl derivative of the methyl ether of the trihydroxyoestrin, in which the two alcoholic hydroxyls are acetylated and the phenolic hydroxyl is methylated, were, however, found to give films stable up to about 7 dynes per cm., and collapse was slow even at higher pressures, so that fairly accurate measurements could be taken above 10 dynes. The results are shown in Figs. 1 and 2.

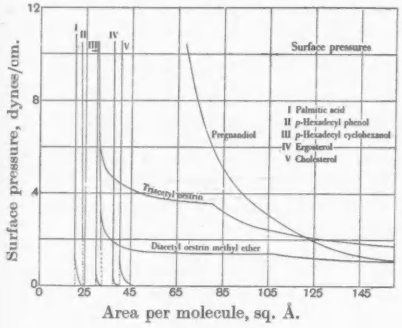


Fig. 1.

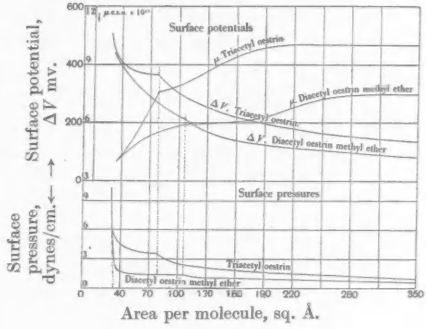


Fig. 2.

Both substances give, at large areas, gaseous films with only moderately large corrections to the perfect gas laws. On compression to about 100 sq. Å., per molecule, a sharp break occurs in the surface pressure area curves, which become nearly, but not quite, horizontal; there is only a slight increase in pressure down to 35 sq. Å. These breaks occur at 81 sq. Å. with the triacetyl compound, and at 109 sq. Å. with the diacetyl methyl ether. The surface pressures at the beginning of this transition region are about 3·6 and 1·5 dynes per cm. respectively; the curves were taken on distilled water and on $N/100$ potassium chloride solution. At the left-hand end of the transition region, the pressure rises sharply, giving a typical curve of a condensed film of the "close-packed head" type, which tends to an area 32·5 sq. Å. at no compression, with a probable error of about 1 sq. Å., for both substances.

These curves resemble, at first sight, the curves for aliphatic substances which give both gaseous and condensed films, but there is a very important difference; in the present case temperature has no detectable influence on the surface pressure at which the transition from gaseous to condensed film takes place, whereas with long-chain substances the surface pressure in the transition region rises steadily with temperature. The change in surface pressure with temperature is less than 0·05 dyne, per 10°, from 0° to 45°.

Fig. 2 shows the changes in surface potential ΔV, due to the film, and also the values of μ, calculated from Helmholtz's equation

$$\Delta V = 4\pi n\mu,$$

n being the number of molecules per sq. cm., and μ the average vertical component of the change in dipole moment of the part of the surface occupied by a molecule of the film, including the dipole moment of the film molecules themselves, and all effects due to redistribution of water molecules and of ions in the underlying solution. The breaks at the points where the gaseous films begin to change into the condensed films are clearly marked on the surface potential curves, at exactly the same areas as on the surface pressure curves. At large areas, although naturally ΔV decreases as the number of molecules in the film becomes smaller, μ increases and becomes practically constant, at 9×10^{-19} e.s.u. per molecule for the triacetyl compound, and $7{\cdot}5 \times 10^{-19}$ e.s.u. for the diacetyl methyl ether. There is a slight decrease in the gaseous film, as the area is reduced, before the discontinuity is reached; at smaller areas the values of μ decrease more rapidly, converging in the case of both compounds to a value about 4×10^{-19} e.s.u. for the condensed film. The surfaces were carefully searched at all areas to see if the surface consisted of two surface phases, the presence of which would be indicated by large fluctuations in ΔV. No fluctuations greater than the experimental error were found, except in the condensed films at pressures where the films were obviously collapsing.

Ultramicroscopic examination disclosed no sign of collapse of the films above about 35 sq. Å.; at smaller areas typical collapse aggregates were seen, forming slowly unless the pressure was above 7 dynes per cm.

The remaining curves in Fig. 1 are inserted for comparison, and include the surface pressure area curves for long-chain phenols [Adam, 1923], cholesterol and ergosterol [Adam and Danielli, unpublished], long-chain *cyclo*hexanols obtained by reduction of the phenols, and the dihydroxylic sterol pregnandiol recently isolated by Marrian [1929] and identified by Butenandt [1931], and for fatty acids on distilled water and other compounds giving the typical curve of closely packed long chains.

The *p*-dodecyl- and hexadecyl-*cyclo*hexanols were specially prepared for this work, in order to obtain an idea of the size of the *cyclo*hexane ring, standing upright and closely packed in the surface. The unreduced benzene ring had earlier been shown to pack to an area of 24 sq. Å. [Adam, 1923]; it was probable that the fully reduced *cyclo*hexane ring would occupy a good deal more space,

partly because of the bulk of the additional hydrogen atoms, and partly because it is a "puckered," not a plane ring. Both the dodecyl- and hexadecyl-*cyclo*hexanols formed condensed films of the "close-packed heads" type, having an area of about 30 sq. Å. at no compression, and being only slightly compressible. The cross section of the oestrin molecule, as packed in the condensed films, is thus about 8 % larger than that of the *cyclo*hexane group. As the temperature was raised, dodecyl*cyclo*hexanol appeared to pass into a liquid expanded film, having an area only slightly greater than that of the condensed film, about 35 sq. Å. at no compression. The investigation of this change was difficult, on account of the unusually small difference in area between the condensed and expanded films; the half expansion temperature under 1·5 dynes compression was $28° \pm 5°$. At high temperatures, the film of hexadecyl*cyclo*hexanol collapsed too rapidly for any expansion to be observed.

Pregnandiol gives a gaseous film at all pressures; the oestrin films resemble those of the *cyclo*hexanol derivatives much more closely than any of the others.

Discussion.

There can be little doubt that the molecules are lying flat, and moving about in the surface more or less independently in the gaseous regions of the films, *i.e.* at areas over 110 sq. Å. per molecule. Previous work has indicated that the molecules are always lying flat in the gaseous state of the films at large areas, though some degree of tilt may be caused by lateral compression. It is equally clear that the molecules are standing on end, closely packed, in the condensed regions of the curves, and therefore the cross-sectional area of the oestrin molecules, in their closest packing, is about 32·5 sq. Å., decidedly less than that of the sterols, and slightly greater than that of *cyclo*hexanol.

It is also certain that the vertical component of the electric moment per molecule is greater when the molecules are lying flat than when they are upright. This must indicate that the direction of the resultant dipole of the various polar groups in the molecule is inclined at a sharp angle to the plane of the molecule as a whole. In all probability this means that the two hydroxyl groups which have an alcoholic character are side by side, on the same side of the ring —*i.e.* they are in *cis*, not *trans*, relation to each other. If they were on opposite sides of the ring, they would neutralise one another. Further, since the dipole component perpendicular to the surface decreases very greatly as the molecules stand upright, it appears that these two hydroxyl groups are arranged with the bond between hydrogen and oxygen, or between oxygen and carbon, at a sharp angle to the plane of the ring.

The transition regions between the condensed and fully gaseous regions of the films indicate varying degrees of tilt in the molecules as the lateral compression is increased. The fact that the vertical dipole component decreases appreciably at areas greater than those corresponding with the discontinuities at 81 and 109 sq. Å., indicates that there is some tilting of the molecules away from the flat position before these areas are reached. There is, however, at the

areas where the discontinuities occur, a sudden decrease in the stability of the molecules lying flat; a comparatively slight increase in lateral compression has a much greater tilting effect here than at greater areas.

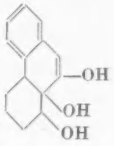
In interpreting the results of surface potential measurements, it must be remembered that μ is not simply the vertical component of the dipole moment of the film molecules, but includes terms of unknown amounts due to re-distribution of ions and re-orientation of water molecules, as Schulman and Rideal [1931] have pointed out. We are not in entire agreement with these authors as to the method of eliminating these secondary effects, and the subject will be discussed by Harding and one of us in another paper; in fact we do not see at the moment any satisfactory way of disentangling the contributions to μ made by the dipole moment of the film molecules themselves and by the re-orientation of the ions and water molecules. This difficulty does not appear, however, to invalidate the above arguments as to the orientation of the dipoles in the molecules; for it is clear that, since the re-orientation of water and redistribution of ions are secondary effects consequent on the introduction of the dipoles of the film molecules at the surface, the total value of μ, including the secondary effects, will increase as the vertical component of the dipole moment of the molecule itself increases.

Confirmatory evidence of the view that the molecules are lying flat in the gaseous state and are tilted at smaller areas, is found in the fact that the pressure required to bring about the transition from gaseous to condensed film is greater with the triacetyl compound than with the diacetyl methyl ether. It is known that acetyl groups have a stronger adhesion to water than methyl ether groups; the acetates of long-chain alcohols form stable films, while the methyl ethers do not [Adam and Dyer, 1924]. The second anchorage, that of the phenolic hydroxyl, would therefore be expected to leave the water surface more easily when methylated than when acetylated. Such a re-orientation as that described above would require the phenolic polar group to leave the water surface. A re-orientation of molecules lying flat, under lateral compression, so that one of two anchorages to the water is broken, is not unknown; it has already been found with several of the long-chain dibasic acids and esters, which have two polar groups, one at each end of the molecule [Adam and Jessop, 1926, 2].

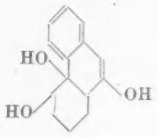
It is clear, since the molecules so easily assume the flat position, that the phenolic hydroxyl is situated near the opposite end of the molecule from the two alcoholic hydroxyls. Butenandt *et al.* [1932] favour the structure (1) or (2). These two structures seem quite impossible when the evidence of surface films is considered, since both (1) and (2) at their areas of closest packing would have a limiting area of about 70 sq. Å. instead of the 32·5 actually found. This small area of 32·5 sq. Å. could only be given by such a structure as (3), with the adjacent alcoholic hydroxyls well to the end of the molecule.

The absence of any effect of temperature on the surface pressure necessary to change the gaseous into the condensed film may perhaps mean that the

molecule is not flexible. One of us has advanced the theory [Adam, 1930, p. 75] that the change from the condensed to the expanded state, in long-chain aliphatic compounds, is due to whip-like oscillations in the flexible hydrocarbon chains. In all cases, where expansion (to either gaseous, vapour expanded, or liquid expanded films) takes place in the aliphatic series, there is a



(1)



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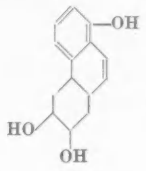

(3)

(4)

(5)

(6)

large temperature coefficient of the pressure in the transition region. The possibility of oscillations in the chains, becoming more violent as the temperature rises, naturally renders the condensed state less stable at higher temperatures. In the case of the oestrin derivatives, we have a change apparently very similar to the expansion of condensed films of aliphatic substances, but without this temperature coefficient. The most probable explanation is that the molecules are not flexible.

Evidence as to the constitution of the molecules.

Skeletons of the phenanthrene, anthracene, naphthacene, chrysene and 2 : 3-benzphenanthrene (4) types among the non-flexible structures appear admissible on the grounds of the area occupied when standing upright. The sterol skeleton of Windaus and Wieland (6) occupies about 36 sq. Å. as a minimum, as is indicated by models, and is therefore excluded. 3 : 4-Benzphenanthrene (5) is probably also too large for an area of 32·5 sq. Å. The structures of a phenanthrene type, in which the lowest ring is standing on one corner, with one of the upper ones slightly to one side, would naturally occupy an area slightly larger than that of the alkyl *cyclo*hexanols; also the anthracene and naphthacene skeletons, in which the rings are all in one line, but the lowest one is lying with a side, instead of a corner, on the water, would be somewhat larger than the *cyclo*hexanols. The inflexibility probably excludes the diphenylbenzene and phenylnaphthalene structures.

The ease with which the molecules lie flat, or stand up, as the lateral compression is changed, indicates that the phenolic hydroxyl is in a remote part

of the molecules from the two alcoholic hydroxyls. Both sets of hydroxyl groups are probably situated near the ends of the molecules. The evidence that the alcoholic hydroxyls are probably in the *cis* position, and inclined at a steep angle to the plane of the rings, has been detailed above.

Preparation of material.

Ketohydroxyoestrin monoacetate. Ketohydroxyoestrin prepared by dehydration of trihydroxyoestrin was heated to 100° for 3 hours with excess of acetic anhydride; excess of water was then added, and the precipitated acetate filtered, washed and dried. It was decolorised by boiling with charcoal in alcoholic solution, and finally recrystallised twice from aqueous methyl alcohol; M.P. 118–121°.

Trihydroxyoestrin monomethyl ether diacetate. Trihydroxyoestrin monomethyl ether was made by treating trihydroxyoestrin in alkaline solution with methyl sulphate in the cold [*cf.* Marrian and Haslewood, 1932]. 30·9 mg. of the methyl ether were heated with 5 cc. acetic anhydride at 100° for 3 hours; excess water was added, the diacetate filtered, washed and dried. It was decolorised with charcoal and crystallised once from aqueous methyl alcohol; M.P. 140–142°.

Trihydroxyoestrin triacetate. Trihydroxyoestrin was heated with excess of acetic anhydride for 3 hours at 100°; excess of water was added, and the acetate filtered off, washed and dried. The product was crystallised twice from aqueous ethyl alcohol; M.P. 119–121°.

p-Dodecyl*cyclo*hexanol and *p*-hexadecyl*cyclo*hexanol were obtained by reducing the corresponding phenols [*cf.* Adam, 1923] in alcoholic solution with hydrogen, under about 2½ atmospheres pressure, using the platinum oxide catalyst of Adams *et al.* [1928]. The products were recrystallised from alcohol.

*Dodecyl*cyclo*hexanol.* M.P. 50–55° (phenol melts at 66°). Iodine value 0. (Found: C, 80·4; H, 13·2 %; calc.: C, 80·6; H, 13·4 %.)

*Hexadecyl*cyclo*hexanol.* M.P. 65–66° (phenol melts at 79°). Iodine value 10. (Found: C, 82·3; H, 13·0 %; calc.: C, 81·6; H, 13·6 %.) The hexadecyl compound may not have been quite fully reduced.

Summary.

Surface pressure and surface potential measurements on diacetyltrihydroxyoestrin methyl ether, and triacetyltrihydroxyoestrin, show that these compounds can orient themselves at a water-air interface, either flat, or perpendicularly to the surface. The vertical component of the change in dipole moment of the surface, per molecule, due to the film, is much greater when the molecules are lying flat than when they are upright. The conclusion is drawn that the molecules probably have an anthracene or phenanthrene skeleton, or a system of four condensed rings, with two alcoholic hydroxyl groups near one end of the molecule, and one phenolic group near the other end. The

alcoholic groups are probably in the *cis* position relative to each other, on adjacent carbon atoms, and are inclined at a steep angle to the plane of the ring in which they are situated.

*Cyclo*hexanols with long chains in the *para*-position to the hydroxyl group give condensed films of area about 30 sq. Å. per molecule, slightly smaller than the cross section of the oestrin molecules.

We are indebted to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants which have defrayed part of the expenses of this research.

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Note added August 17th. Since this paper was written, Marrian and Haslewood [*Lancet*, 1932, ii, 282] have shown that no double linkage other than those in the benzene nucleus is present in the oestrin molecule. This indicates that the molecule must have a four-ringed structure. Butenandt (*Nature*, August 13th) has now advanced similar views.

CXLVII. THE DETERMINATION OF SMALL QUANTITIES OF OXALIC ACID.

By EDWARD CHARLES DODDS and ERIC JAMES GALLIMORE[1].

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(*Received June 28th, 1932.*)

The determination of small amounts of pure oxalic acid in aqueous solution by precipitation of the calcium salt and treatment of the precipitate with permanganate offers little difficulty. In the procedure elaborated by Leulier [1929] quantities of oxalic acid as low as 0·05 mg. in aqueous solution may be estimated with accuracy.

The lack of success which has attended the efforts to estimate oxalic acid in biological materials is due to the difficulty of obtaining the oxalic acid in a solution free from those substances which either inhibit the precipitation of calcium oxalate or which are carried down with the precipitate and react with the permanganate.

Investigations on the rôle of oxalic acid in metabolism have for the most part been based on the determination of oxalic acid in urine. It is not within the scope of this paper to discuss the literature in detail but a brief account of the more important investigations is given below. Salkowski [1900] estimated oxalic acid in urine by extracting the acidified urine with a mixture of alcohol and ether, afterwards precipitating the extracted oxalic acid as the calcium salt and weighing the calcium oxide formed by ignition. Autenrieth and Barth [1902] precipitated the oxalic acid directly from the urine as calcium oxalate and then transferred the oxalic acid from an acid solution into a mixture of alcohol and ether; the oxalic acid was again precipitated as calcium oxalate and the precipitate after ignition was weighed. Dakin [1907] modified the method of Autenrieth and Barth by using ether, without alcohol, in a continuous extractor. The final precipitate was either titrated with permanganate or weighed after ignition. Merz and Maugeri [1931] give references to adverse criticisms of the above methods and state that they were unable to extract oxalic acid quantitatively from its aqueous solution with ether. Van Slyke and Sendroy [1929] have described the advantages of determining small quantities of oxalic acid by a gasometric method in lieu of the many titration methods which have been used.

[1] Whole-time worker for the Medical Research Council.

Experimental.

The method of estimating oxalic acid by direct precipitation of the calcium salt from urine was found to give results both low and variable.

With regard to the extraction of oxalic acid from aqueous solution with ether, it was found that the extraction was incomplete with pure oxalic acid but with a high concentration of free mineral acid all the oxalic acid could be removed by this means. When applied to urine the ether also extracts from the strongly acidified concentrated urine many other substances, the presence of which, even though the calcium oxalate is precipitated from an alkaline, and reprecipitated from an acid, solution, may cause anomalous results.

The method about to be described depends on the fact that it was found possible to remove oxalic acid completely by esterification and distillation *in vacuo* from an acid alcoholic solution whilst apparently leaving behind all interfering substances.

Reagents. Alcohol, containing dry HCl, *N* solution. Lime-water, saturated solution, free from carbonate. Ammonium phosphate, 0·5 %.

Distillation of oxalic acid. 25 cc. of urine + 1 cc. *N* HCl are evaporated almost to dryness in a Claisen flask *in vacuo* below 50°. The flask is then connected up as shown in Fig. 1.

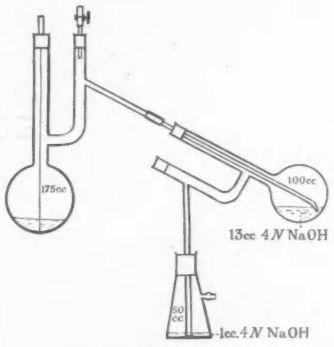

Fig. 1.

The receiver contains 13 cc. 4*N* NaOH and is kept cool by means of a rapid stream of cold water. To the residue in the flask a mixture of 5 cc. alcohol and 2 cc. benzene is added, the temperature of the water-bath surrounding the distillation flask raised to 65° and the contents of the flask dried by distilling off *in vacuo* the low boiling ternary mixture of alcohol, benzene and water. To remove the last traces of water, it is again distilled with 5 cc. acid alcohol and 2 cc. benzene.

The contents of the flask are now boiled with 20 cc. of the alcohol containing HCl on a boiling water-bath for half an hour; the flask is then reconnected to the receiver and the contents distilled *in vacuo* at 65°. The vacuum reached

about the middle of the distillation should not be less than 30 mm. The boiling and distillation are repeated in all three times.

The contents of the Büchner flask are washed into the smaller Claisen flask and the slightly alkaline solution evaporated down to 10–20 cc. *in vacuo*.

Treatment of the distillate. To free the oxalic acid from the high concentration of sodium chloride, an excess of 2 cc. 5*N* HCl is added and the solution extracted with ether in a continuous extractor for six hours. To the ether solution 5 cc. of water are added and the ether removed by distillation. The solution is filtered if necessary, made alkaline to methyl red with *N*/10 NaOH and concentrated to about 3 cc. on a water-bath.

Precipitation of the oxalic acid. The solution of oxalic acid in 3–5 cc. water in a 15 cc. centrifuge-tube is faintly acidified with *N*/10 acetic acid and mixed with 2 cc. of saturated lime-water. After standing 12 hours at 0° 0·5 cc. of ammonium phosphate is added. The precipitate is centrifuged, decanted and washed once with 2 cc. of water.

The oxalic acid in the precipitate is estimated by the gasometric method of Van Slyke and Sendroy [1929].

Results.

The following are some results on urine and on urine with added oxalic acid.

Table I.

	mg. $(COOH)_2$ found	mg. $(COOH)_2$ recovered	% error
25 cc. Urine I	0·47, 0·43 } 0·45		
" "			
25 cc. Urine I + 1 mg. $(COOH)_2$	1·36	0·91	−9
25 cc. Urine I + 0·5 mg. $(COOH)_2$	0·96	0·51	+2
25 cc. Urine II	0·39		
25 cc. Urine II + 0·3 mg. $(COOH)_2$	0·69	0·30	0
25 cc. Urine III	0·55, 0·57 } 0·56		
" "			
25 cc. Urine III + 0·3 mg. $(COOH)_2$	0·87	0·31	+3
25 cc. Urine III + 0·5 mg. $(COOH)_2$	1·08	0·52	+4
25 cc. Urine III + 1·0 mg. $(COOH)_2$	1·54	0·98	−2

Comparison with results obtained by ether-extraction of the urine.

250 cc. urine III were heated for twenty minutes on a boiling water-bath with 10 cc. of 10*N* HCl and concentrated *in vacuo* to 100 cc. A further 10 cc. of 10*N* HCl were added and the concentrated urine was extracted with ether for 30 hours in a continuous extractor fitted with a stirrer. After adding 5 cc. water the ether was distilled off. Strong ammonia was added to the residue to give an approximately 2*N* solution of ammonia and the contents of the flask were washed into a 15 cc. centrifuge-tube. 2 cc. *M* $CaCl_2$ were added and the precipitate left overnight, centrifuged, decanted, the precipitate washed once

with 3 cc. 8*N* ammonia and once with 3 cc. water, stirring the precipitate up with a thin rod at each washing. The precipitate was then heated with 3 cc. *N* HCl, filtered and washed with a further 2 cc. of *N* HCl, and the filtrate was diluted with a few cc. of water. The clear solution was made alkaline to methyl red with ammonia, 0·5 cc. *M* $CaCl_2$ added and *N* acetic acid till faintly acid. After standing overnight it was centrifuged, decanted, and the precipitate washed twice, each time with 2 cc. of distilled water. The precipitate was dissolved in sulphuric acid as in the Van Slyke method, and the solution transferred to a 10 cc. flask. Aliquot portions of the solution were taken for the gasometric determination.

Duplicate results gave an oxalic acid content in the 250 cc. taken of 4·9 and 5·2 mg. respectively, appreciably lower than the content as determined by distillation (Table I).

The principle of the method has also been applied to the determination of oxalic acid in blood, from which small quantities of added oxalic acid have been satisfactorily recovered. The proteins are removed by phosphotungstic acid. Further details of this work, together with a series of determinations, will be reported in a future communication.

Summary.

A method is described whereby oxalic acid can be quantitatively estimated. By a preliminary esterification and distillation the oxalic acid is freed from interfering substances and can then be precipitated as calcium oxalate and the oxalic acid in the precipitate estimated gasometrically.

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CXLVIII. A COLORIMETRIC METHOD FOR THE DIRECT DETERMINATION OF UREA IN URINE.

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(*Received June 30th, 1932.*)

Since Fosse [1914] introduced a gravimetric method for the estimation of urea in urine based on the precipitation of urea as dixanthylurea, no attempt has been made to apply the principle to meet the requirements of the routine laboratory analyst who has many estimations to perform in a limited time. The purpose of this paper is to present a method suitable for use in the routine laboratory.

The tedious process of collecting, washing and weighing the dixanthylurea has been replaced by the separation of the precipitate by centrifuging; the nitrogen content of the substance is determined by a colorimetric estimation of the ammonia produced in a micro-Kjeldahl digestion, which has been facilitated by the introduction of a modified digestion reagent containing selenium, instead of copper, as catalyst [Lauro, 1931], and perchloric acid instead of sulphuric acid as the oxidising agent. The advantages of perchloric acid are mentioned in a recent paper by King [1932], who uses this acid for the estimation of total phosphorus in blood. The whole process may be carried out in the same tube. The difficulty of sedimentation of the light flocculent precipitate of dixanthylurea has been overcome by precipitating the dixanthylurea in the presence of a very fine suspension of barium sulphate, when the two precipitates centrifuge down together leaving a clear supernatant liquid which may be safely decanted.

Proteins and interfering substances are removed from the urine by adsorption on "colloidal iron" which is produced in the liquid by the interaction of dilute ferric chloride and sodium bicarbonate solutions.

Method.

Reagents.

(1) 1 % crystalline ferric chloride in water.

(2) 0·5 % sodium bicarbonate in water.

(3) Barium reagent. Dissolve 10 g. of sodium chloride and 2 g. of sodium sulphate in 90 cc. of distilled water. Add this solution to 400 cc. of glacial

acetic acid, and to the mixture add 1·2 g. of barium chloride dissolved in 10 cc. of water.

(4) 2·5 % solution of xanthydrol (Schering-Kahlbaum) in glacial acetic acid. This solution should be kept in a small well-stoppered bottle and prepared every few days.

(5) Digestion mixture:

100 cc. of 60 % perchloric acid;
40 cc. of phosphoric acid (sp. gr. 1·75).
20 cc. of 3 % sodium selenate solution;
0·5 cc. of 10 % sodium sulphate solution.
130 cc. of water.

(6) Nessler reagent [Folin and Wu, 1919].

(7) Ammonium sulphate solution. Dissolve 0·1179 g. of pure ammonium sulphate in water. Preserve from moulds with two drops of strong sulphuric acid and dilute to 1 litre.

Procedure. Measure 1 cc. (or 2 cc. if the urea content is very low) of urine from an Ostwald pipette into a 100 cc. graduated flask, add 2 cc. of 1 % ferric chloride and, while rotating the liquid, 10 cc. of 0·5 % sodium bicarbonate solution. Dilute to the mark, mix and filter. Transfer a suitable amount of filtrate (1–5 cc. depending upon the urea content) with an Ostwald pipette to a 4″×1″ hard glass round-bottomed centrifuge-tube[1] of 35–40 cc. capacity. Table I has proved useful in choosing the volume of filtrate required, but it

Table I.

Specific gravity of specimen	cc. of filtrate required
1030–1025	1
1025–1020	1·5
1020–1015	2
1015–1010	3
1010–1005	5
Below 1005	Use 2 cc. of urine for filtration

must be borne in mind that other factors beside the urea content affect the specific gravity of a urine.

Dilute the filtrate in the centrifuge-tube to 5 cc. with water, add 5 cc. of well mixed barium reagent and 1 cc. of xanthydrol solution. Mix the liquid, and allow the tube to stand in a bath of ice-cold water for 20 minutes. Mix the liquid by rotating the tube; wash down the sides of the tube with 1 cc. acetone, which is allowed to float on the surface. Centrifuge for five minutes and decant the supernatant liquid, placing the inverted tube on a sheet of filter-paper for two minutes to drain. Remove the excess liquid from the inside of the tube by wiping with filter-paper.

Add 1 cc. of digestion mixture and a piece of porous pot. Heat the tube gently on a sand-bath in a fume chamber, covering the mouth of the tube with a watch-

[1] Supplied by C. Dixon and Co., 27 Devonshire Street, W.C. 1.

glass when heavy white fumes appear and the liquid darkens. Twenty seconds after the brown-coloured liquid becomes colourless remove the tube from the sand-bath. Add 20 cc. of water after the acid has cooled for a few minutes. Cool to room temperature and add 7·5 cc. of Nessler reagent while twirling the liquid in the tube. After mixing, centrifuge for three minutes to remove the precipitate of barium sulphate. Compare the crystal clear supernatant liquid with the standard which is prepared at the same time as follows. Place 5 cc. of barium reagent and 5 cc. of water *etc.* in a 4″×1″ tube, and centrifuge, drain and digest as described for the unknown. After heating, add 15 cc. of water and 5 cc. of standard ammonium sulphate solution before adding 7·5 cc. of Nessler reagent.

The use of a thick blue glass light filter for the comparison in the colorimeter is recommended, since by this means the difficulty always associated with the matching of yellow or brownish-yellow colours is eliminated; the red tints which result from the use of the blue glass being easily compared.

Calculation (if the standard is set at 20 mm.):

$$\frac{53{\cdot}6}{\text{Colorimetric reading} \times \text{number of cc. filtrate used}} = \text{g. of urea per 100 cc. of urine.}$$

Table II, which contains results taken at random from many analyses, gives the experimental values found by the analysis of standard urea solutions.

Table II.

Concentration of solution taken in g. per 100 cc. %	Experimental result in g. per 100 cc. %	Percentage error
4·2	4·23	+1
3·0	3·05	+1·5
2·8	2·73	−2·5
2·0	1·98	−1
1·5	1·48	−1
1·0	0·98	−2
0·75	0·73	−2·5
		Mean error 1·6

The recovery of urea added to urine is shown in Table III.

Table III.

% urea in urine	% urea added	Total urea %	% urea found
0·81	1·0	1·81	1·82
0·65	1·0	1·65	1·67
0·85	1·5	2·35	2·32
4·21	0·75	4·96	5·00
0·29	1·5	1·79	1·75
1·58	1·0	2·58	2·55

The close agreement between the xanthydrol and urease methods when applied to a number of specimens of urine is shown in Table IV. The direct titrimetric method of Cole [1931] was employed for the estimation of urea by the urease method.

Table IV. *Comparison of results obtained by urease and xanthydrol methods.*

Result by xanthydrol method (g./100 cc.)	Result by urease method (g./100 cc.)
1·55	1·56
1·19	1·18
0·54	0·55
1·53	1·48
0·57	0·56
1·57	1·55
2·31	2·27
2·80	2·81
3·15	3·08
0·72	0·71
2·76	2·78
1·38	1·35

Discussion.

Twelve urines were examined in order to demonstrate the absence of adsorption of any nitrogen-containing substance on the barium sulphate. To illustrate this, thirty times the amounts of reagents and urine filtrate normally taken were used, xanthydrol not being added. After the precipitate of barium sulphate was spun down and subjected to the usual treatment, no appreciable colour developed when the liquid was treated with Nessler reagent.

The digestion is free from difficulty, as owing to the small volume of liquid which has to be removed by boiling, bumping is practically unknown. The end of the digestion is clearly defined, the liquid changing from brown to colourless in a few seconds.

It is unnecessary to wash the precipitate of dixanthylurea as the amount of urea-free liquid left in the tube after the treatment described is of the order of 0·1 cc. If desired, the tubes may be calibrated at 30 cc. and the contents of the tube diluted to this mark, but this is not essential.

The ferric chloride and sodium bicarbonate solutions when used in the amounts stated will remove up to 2·5 % of protein from urine. That no adsorption of urea takes place during the precipitation was shown by treating a mixture of 1 cc. of 0·6 % urea solution and $\frac{1}{3}$ cc. of plasma (containing 40 mg./100 cc. of urea) with the protein precipitants before diluting to 100 cc. The urea content of the filtrate was found to be 0·0061 %.

We are at present engaged in applying the principle to the determination of urea in blood-filtrates.

Summary.

A method is described for the routine estimation of urea in urine based on the formation of dixanthylurea. Interfering substances are removed from the urine with ferric chloride and sodium bicarbonate, and the urea is precipitated from the filtrate by xanthydrol. The precipitate is centrifuged in the presence of barium sulphate and its nitrogen content determined colorimetrically after a micro-Kjeldahl digestion.

We wish to express our thanks to Prof. E. C. Dodds for his help and advice concerning a number of problems which arose in the course of this work, and to Dr T. Bloem for his kind assistance.

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CXLIX. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

VI. THE CONVERSION OF FRUCTOSE AND GLUCOSE TO LACTIC ACID BY EMBRYONIC TISSUES.

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(*Received June 30th, 1932.*)

Fructose is known to be utilised by certain animal and vegetable cells; thus it is fermented at least as rapidly as glucose by yeast-juice [Harden and Young, 1908; 1909] and certain yeasts [Slator, 1908; Willstätter and Sobotka, 1922; Fernbach, Schoen and Mori, 1927; Hopkins, 1931]; it is converted into lactic acid by tumours though to a less extent than glucose [Minami, 1923; Warburg, Posener and Negelein, 1924; Rosenthal, 1930, 1]; whilst in the summer months minced frog's muscle [Laquer and Meyer, 1923] and rat-liver [Rosenthal, 1930, 2] form lactic acid from fructose more rapidly than from glucose. On the other hand, brain tissue [Loebel, 1925] and nerve tissue [Gerard and Meyerhof, 1927] cannot convert fructose into lactic acid. The action of the other tissues has apparently hitherto not been investigated. Some preliminary experiments by the authors, however, indicate that with kidney, spleen, testis and retina there is little anaerobic attack on fructose.

Whilst fructose is not in general present in any considerable amount in the body, it has been known since the time of Claude Bernard that fructose normally occurs in the pregnant animal of several species, attaining a high concentration (up to *ca.* 1 %) in the amniotic and allantoic fluids and in the foetal blood [Bernard, 1850; Dastre, 1876; Gürber and Grünbaum, 1904; Paton, Watson and Kerr, 1907; Orr, 1924]. Therefore, it becomes of special interest to find out if fructose is utilised by embryonic tissues, which are in many cases bathed in a medium rich in this sugar. In the present paper the anaerobic conversion of fructose and glucose into lactic acid by the different embryonic tissues of several species is described.

The work of Warburg and his collaborators [Warburg, 1926, 1930] has shown the extraordinary ability of embryonic tissues to form lactic acid from glucose, the foetal membranes of the rat resembling the most active tumours in this respect. It may be noted, however, that almost all these investigations refer to the rat, and therefore the figures which we give for other species are of interest.

It will be shown that while glucose is converted readily into lactic acid by all the embryonic tissues, the attack on fructose depends both on the tissue and on the species, varying from zero to a value equal to that observed with the same tissue on glucose.

The discussion of the results follows the experimental section.

Nomenclature. For the sake of brevity, we shall use the following terms.

Glycolysis.	General expression denoting the breakdown of carbohydrate to lactic acid [*cf.* Warburg, 1925].
Glucolysis.	The glycolysis in the presence of added glucose.
Fructolysis.	The glycolysis in the presence of added fructose.
Autoglycolysis.	The glycolysis when no substrate is added.

This distinction between glycolysis and glucolysis is in conformity with the officially approved nomenclature now adopted for the glycosides [Armstrong and Armstrong, 1931].

EXPERIMENTAL.

Preparation of material and methods used.

The smaller animals were killed by a blow on the neck; the larger ones were killed under ether anaesthesia. The animal was immediately immersed in warm, dilute lysol, and after a few minutes was washed thoroughly with running water. The uterus was then removed and dissected with sterile instruments.

After dissection, portions of the embryonic membranes of suitable size were cut with scissors: such material is particularly suitable for *in vitro* experiment since it is sufficiently thin to be used directly without further cutting. The placenta was used intact when it was very small, otherwise it was cut with a razor into thin slices. In all cases the material was rinsed in a dish containing Ringer solution before being transferred to the manometer vessels, where the tissues were suspended in Ringer solution containing 25 *m.M.* per litre of sodium bicarbonate and saturated with nitrogen containing 4·5–5 % CO_2 by volume (p_H 7·45–7·50). Sugar was added to the Ringer solution in freshly prepared 10 % solution to give in all cases a final concentration of 0·2 %. The fructose used was Schering-Kahlbaum "Laevulose cryst. for scientific purposes." Recent analyses of this product have been made by Wierzuchowski *et al.* [1931]; polarimetric measurements on the specimen used by us agree with those given by these authors.

The glycolysis was measured manometrically at 38° by the method of Warburg [1926; 1930]. The first readings were taken 10 to 15 minutes after putting the vessels in the bath, and were usually continued for a period of 2 to 3 hours. These prolonged experiments were desirable since we have found that the autoglycolysis of embryonic tissues is often large in the first hour of the experiment. The general procedure was to make simultaneous observations of the glycolysis of pieces of the same tissue in glucose, fructose and sugar-free Ringer solutions.

In typical cases (rat-yolk-sac and cat-amnion) the lactic acid formation

measured manometrically was confirmed by chemical estimation by Clausen's method. The results show that the action of the embryonic membranes on fructose is a pure lactic acid fermentation.

The glycolysis is expressed throughout by Warburg's quotient $Q_M^{N_2}$, which is defined as the number of mm.^{3} of CO_2 at N.T.P. liberated per hour per mg. dry weight of tissue. Since 1 gram-equivalent of lactic acid liberates 22·4 litres of CO_2, the lactic acid per hour formed is expressed as a percentage of the dry weight of the tissue when these figures are multiplied by 0·4.

The disposition and development of the embryonic membranes in the several species are so varied that in the following text we quote references to standard works for the description of the individual membranes as they arise. The experiments are numbered with respect to the individual animals; experiments bearing the same number thus refer to tissues of the same animal throughout.

Glycolysis of the foetal membranes.

Outer sac.

The results are collected in Table I.

Although for the sake of convenience we describe together the outer membranes of the various species, it must be remembered that the outer sac of Rodentia consists of the inverted inner half of the yolk-sac, the outer half of the yolk-sac having disappeared together with the greater part of the chorion laeve during the second half of pregnancy [Grosser, 1927, p. 122]. On the other hand, the outer sac of Carnivora [Grosser, 1909, pp. 131–2] consists of the chorion laeve in close contact with the outer wall of the allantois.

Rodentia.

Rat (*albino*). (For description of membranes see Grosser [1927, p. 135].) The inner and outer membranes were dissected from the embryo and placenta, and the yellowish yolk-sac carefully freed from the fine white amnion. Pregnancies of known date are readily obtained in this species from observations of the occurrence of vaginal plugs. Observations on the glucolysis, fructolysis and autoglycolysis of the yolk-sac of rats 13 to 20 days pregnant (*i.e.* in the second half of pregnancy) are given. The glucolysis remained practically constant for the 3 hours of the experiment, the Q values agreeing well with those obtained in Ringer solution by Kumanomido [1928]. The fructolysis is of the same order of magnitude as the glucolysis (in Exp. 7 the values were identical for the 2 hours of the experiment), but in no case did the fructolysis exceed the glucolysis. In Exps. 4 and 7 the fructolysis and glucolysis ran parallel, in the remainder the fructolysis fell off more rapidly than the glucolysis. The autoglycolysis was sometimes appreciable in the first 30 minutes, but afterwards fell to a negligible value.

Mouse [Jenkinson, 1913, p. 232]. Whilst the glycolysis of the mouse-yolk-

Table I. *Outer membrane.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1 hours	Glucolysis 2 hours	Glucolysis 3 hours	Fructolysis 1 hours	Fructolysis 2 hours	Fructolysis 3 hours	Autoglycolysis 1 hours	Autoglycolysis 2 hours	Autoglycolysis 3 hours	Embryo Wt. g.	Embryo Age days	Relative fructolysis 2nd hr. %
Rat (albino)												
4	35·5	32·5	31·5	27·0	26·5	27·5	1	0	0	0·065	13	82
3	32·0	27·5	27·0	30·0	22·0	20·0	4	1	0·5	0·175	15	79
				28·0	22·0	19·0	—	—	—	—	—	79
				30·0	22·0	—	—	—	—	—	—	79
8	24·2	24·0	21·4	21·2	18·8	13·7	—	—	—	0·34	16	—
35	18·0	17·0	17·5	11·5	9·0	8·5	4	1	1	2·6	20	50
1	24·0	19·0	18·0	18·0	13·0	11·0	7	1·5	0	3·2	20	66
7	19·0	16·0	—	20·0	15·5	—	2·5	0	—	3·2	20	97
				20·0	15·0	—	—	—	—	—	—	94
				20·0	16·0	—	—	—	—	—	—	100
											Mean	75
Mouse												
27	26·2	22·8	—	7·0	4·0	—	7·2	2·3	—	0·425	15	8
33	24·2	20·3	—	5·8	3·7	—	6·7	1·1	—	0·62	16	13·5
42	26·0	19·0	—	9·5	4·5	—	9·0	2·0	—	1·0	17	15
											Mean	12
Guinea-pig												
18	11·2	*11·2*	—	11·9	*11·6*	—	8·4	*3·2*	—	30	50	105
29	11·0	11·0	—	10·0	9·5	—	2·0	0·5	—	31·5	50	86
											Mean	96
Rabbit												
26	25·5	25·0	—	12·0	10·0	—	6·0	1·0	—	0·07	13	37
24	17·0	15·0	—	9·5	7·5	—	4·5	1·0	—	30·2	27	46
22	22·4	23·1	—	5·5	4·4	—	3·0	1·1	—	31·6	27	15
											Mean	33
Cat												
11	*41·0*	*24·0*	—	*6·0*	*5·0*	—	*5·0*	*5·0*	—	19·5	—	0
38	16·5	13·5	13·0	3·0	2·5	1·5	2·0	1·0	1·0	45·5	—	12
13	17·0	15·0	—	4·8	2·8	—	2·9	1·8	—	46·0	—	7·5
36	17·0	14·4	—	4·1	2·2	—	3·3	1·4	—	58·5	—	6
10	17·0	10·5	—	3·0	2·5	—	3·5	2·0	—	109	—	6
											Mean	6
Ferret												
23	34·5	31·5	—	—	—	—	4·0	1·3	—	1·4	—	—
19	*29·0*	*27·0*	—	*6·0*	*5·0*	—	*6·0*	*4·0*	—	2·9	—	4
34	29·0	23·0	19·5	8·0	7·0	6·0	3·0	0·5	0·3	6·0	—	29
				8·5	6·0	5·5	—	—	—	—	—	24
											Mean	15·5
Human peripheral membrane												
Maternal side												
17	9·3	8·0	—	6·3	4·6	—	2·9	0·8	—	10 months		53
21	8·0	6·3	—	4·2	2·4	—	2·3	1·0	—	—	—	26
28	8·2	7·9	—	7·2	6·0	—	2·1	0·8	—	—	—	73
											Mean	51
Foetal side												
17	3·8	1·9	—	4·1	0·6	—	3·8	0	—	—	—	32
20	3·9	*3·0*	—	3·6	*2·6*	—	2·5	*1·4*	—	—	—	75
28	6·5	6·0	—	4·3	2·9	—	1·2	0·7	—	—	—	42
											Mean	50

The figures given in italics in these Tables are the values of $Q_M^{N_2}$ calculated from half-hour experimental periods. The ages given are calculated from the weights, using the tables of Needham [1931, Appendix I]: in the rats the ages were known, and agreed with those calculated.

sac is shown to be comparable with that of the rat, the fructolysis on the other hand is negligible, barely exceeding the autoglycolysis.

Guinea-pig [Grosser, 1927, pp. 38, 148]. Values for two late pregnancies are given. Although the glucolysis and fructolysis are less than those of the rat at a correspondingly late stage (Exps. 1 and 7, Table I), the fructolysis resembles that of the rat in being practically identical with the glucolysis.

Rabbit [Grosser, 1927, p. 122]. Results for two very advanced pregnancies and for one half-way through gestation are included in Table I. The glucolysis is of the same order as that of the rat-yolk-sac, but the membrane differs from the yolk-sac of the other rodents investigated in that it attacks fructose at about one-third of the rate at which it converts glucose to lactic acid. The fructolysis as well as the glucolysis remains constant over the experimental period.

Carnivora.

Cat. The allanto-chorion of the cat was used without attempting to separate it into its component layers, although it was noted that such a separation occurred partially during the course of the experiment. The membrane is not inferior to that of the rat in attacking glucose, but the fructolysis is negligible, being only slightly greater than the autoglycolysis.

Ferret. The allanto-chorion of the ferret was used in the same way as that of the cat. The glucolysis is at least as high as that of the rat; the fructolysis, while steady and relatively greater than that of the cat, is still small.

Man. The human material used in this paper was obtained from the full-time afterbirth only, and was used within half an hour of its expulsion, while the placenta was still warm. It was kindly supplied by the Obstetric Department of the Middlesex Hospital.

The membrane attached peripherally to the placenta of the afterbirth consists, after separation of the amnion, of chorion laeve together with decidua [Grosser, 1909, pp. 269, 212]. As this membrane was thick, it was divided. On pulling apart, it separated fairly easily along a plane nearer to the foetal side into foetal and maternal portions, which were used separately in the manometers. Histological examination indicated that the cleavage had occurred through the "Zwischenzone" [Grosser, 1909, p. 212] which consists of chorionic epithelium and the remains of villi, decidua capsularis and decidua compacta. The foetal portion thus consisted largely of chorionic connective tissue together with a small amount of the "Zwischenzone"; the maternal part consisted largely of the latter. As was to be expected from the known low metabolic activity of connective tissue, it was found (Table I) that the metabolism of the maternal portion was higher than that of the foetal. Both attacked fructose, but to about half the extent to which they attacked glucose.

Amnion.

The glycolysis of the amnion of the rat, rabbit, guinea-pig, cat and ferret in glucose, fructose and sugar-free solutions is shown in Table II. The preparation of the membrane presented no difficulties; in the rabbit, cat and

Table II. *Amnion.* $Q^{N_2}_M$.

Exp. no.	Glucolysis 1	2	3	Fructolysis 1	2	3	Autoglycolysis 1	2	3	Embryo Wt. g.	Age days	Relative fructolysis 2nd hr. %
	hours			hours			hours					
Rat (albino)												
32	16·0	16·0	16·0	5·0	3·0	2·0	6·0	0	0	1·6	19	19
35	15·0	14·5	14·5	7·0	3·5	4·0	—	—	—	2·6	20	Not > 24
7	17·0	11·5	—	7·0	2·0	—	—	—	—	3·2	20	Not > 17
												Mean 19
Rabbit												
37	17·5	13·5	14·0	5·5	4·5	4·5	1·5	0·5	0	36·5	28	31
				6·5	5·0	5·5	—	—	—	—	—	35
												Mean 33
Guinea-pig												
18	5·1	*5·2*	—	3·8	*2·6*	—	2·8	*0·2*	—	30·0	50	48
29	5·8	6·0	—	4·0	2·5	—	2·6	0·4	—	31·5	50	38
												Mean 43
Cat												
11	*17·0*	*11·0*	—	*10·0*	*8·0*	—	*4·0*	*4·0*	—	19·5	—	57
13	15·7	15·0	—	6·9	7·5	—	3·8	1·9	—	46·0	—	43
41	—	—	—	10·6	8·2	8·0	—	—	—	57·0	—	—
				14·6	10·3	9·8	—	—	—	—	—	—
36	12·5	12·5	—	6·5	7·5	—	6·0	4·5	—	58·5	—	37
												Mean 46
Ferret												
23	15·5	13·5	*13·0*	13·5	13·0	*12·0*	10·5	1·0	0	1·4	—	96
19	*18·4*	*13·2*	—	*12·8*	*10·4*	—	*8·2*	*1·6*	—	2·9	—	76
												Mean 86
Rabbit-allantois												
24	22·0	14·2	—	16·0	11·5	—	12·0	2·5	—	30·2	27	77
37	19·0	20·0	20·0	13·0	11·5	11·5	1·0	0·5	0	36·5	28	56
				12·0	12·5	11·5	—	—	—	—	—	62
												Mean 68
Cat-yolk-sac												
38	21·0	19·5	17·5	11·5	9·5	8·5	5·0	2·5	1·5	45·5	—	41
				11·0	9·5	10·5	—	—	—	—	—	41

ferret, dorsal portions were taken in order to avoid free allantois. The membrane in the cat and ferret consists of fused allantois and amnion [Grosser, 1909, p. 132]. The glucolysis was in all cases steady over the course of the experiment. It should be noted that the values obtained for the rat were from late pregnancies, and therefore were lower than the value in horse-serum obtained from an early pregnancy by Negelein [1925]. The fructolysis of amnion of the rat is small compared with the glucolysis; that of the ferret approaches the glucolysis; whilst in the rabbit, guinea-pig and cat the amnion is intermediate in its activity towards fructose.

The metabolism of the amnion from the human afterbirth at term was

found to be very low. This is scarcely surprising since the amnion undergoes a hyaline degeneration at the end of pregnancy, which Belosor [1925] considers to be a physiological preparation for its rupture.

Allantois.

Measurements on the allanto-chorion and allanto-amnion of the Carnivora have been described above. Free allantois can be obtained in sufficient quantity from the rabbit. In the later stages the allantoic sac stretches between the placenta and the ventral surface of the embryo, over which it is fused with the amnion [Grosser, 1927, p. 129]. The side portions consist of pure allantois and are easily dissected. The results are given in Table II, from which it will be seen that the glucolysis is high and almost identical with that of the yolk-sac of the same animal (Exp. 24). The allantois readily attacks fructose, to a greater extent than either the amnion or the yolk-sac of the same animal. The good agreement between the duplicates in fructose shows that the results are highly reproducible and the membrane homogeneous.

Yolk-sac.

The modified yolk-sac of Rodentia, which takes on the special nutritive function of the primary chorion laeve [Grosser, 1927, p. 84] and even develops villi in the later stages [Robinson, 1892], has been studied above. In Carnivora, the yolk-sac persists throughout pregnancy as a small intact sac, presumably with very different function from that of the rodents. The cat was the most convenient source of this type of yolk-sac. In this animal it is found as a yellow sac lying between embryo and placenta and drawn out into two long horns in the direction of the long axis of the uterus. It was easily dissected and freed from allantois. The results are given in Table II. It was found to attack glucose slightly more readily than the allanto-chorion or allanto-amnion from cat embryos of the same age. The fructolysis was a little less than half the glucolysis, and remained constant. Here again the uniformity of the material is shown by the equal fructolysis of two portions from different parts of the membrane.

PLACENTA.

Previous studies have shown that the placenta of the rat [Murphy and Hawkins, 1925; Loeser, 1927], mouse [Fujita, 1928], rabbit [Bell *et al.*, 1928] and man [Loeser, 1927; Bell *et al.*, 1928; Kumanomido, 1928] readily converts glucose into lactic acid. These workers all used either the whole organ or slices cut from it; placenta *Brei* has been studied by Ishikawa [1928] (rabbit) and Hayashi [1928] (human), who differ as to whether added hexoses are converted into lactic acid at all by the placenta.

We have studied the glucolysis, fructolysis and autoglycolysis of the placenta of the rat, mouse, guinea-pig, rabbit, cat, ferret and human being (Table III).

Table III. *Placenta.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1	Glucolysis 2	Glucolysis 3	Fructolysis 1	Fructolysis 2	Fructolysis 3	Auto-glycolysis 1	Auto-glycolysis 2	Auto-glycolysis 3	Embryo Wt. g.	Embryo Age days	Relative fructolysis %	Remarks
	hours			hours			hours						
Rat													
16	*17·2*	*12·8*	—	*12·8*	*5·6*	—	*9·8*	*5·2*	—	0·03	13–14	5	Whole placenta
8	13·2	11·3	11·7	6·5	3·5	1·6	4·1	2·9	1·8	0·34	16	7	—
32	19·5	14	14	8	3	2·5	7·5	1·5	2	1·6	18–19	12	—
1	16·5	15·5	15	4·0	2·5	2·0	3·5	2·0	2·0	3·2	20	4	—
											Mean	7	
Mouse													
27	7·0	6·5	—	4·8	3·4	—	5·3	3·1	—	0·425	15	9	Materna half
27	8·3	8·0	—	2·6	2·1	—	3·5	2·0	—			2	Foetal half
33	7·2	7·3	—	2·4	1·7	—	2·7	1·3	—	0·62	16	7	—
											Mean	6	
Guinea-pig													
18	5·1	*4·2*	—	3·3	*1·4*	—	2·5	*1·2*	—	30	50	7	—
29	4·25	3·15	—	2·1	0·9	—	1·8	0·8	—	31·5	50	4	—
											Mean	6	
Rabbit													
26	9·6	7·2	—	4·5	2·2	—	6·6	4	—	0·072	14	0	—
24	4·6	3·4	—	1·9	1·5	—	1·95	1·0	—	30·2	27	21	—
22	4·6	3·9	—	2·3	1·5	—	1·7	0·9	—	31·6	27	20	—
											Mean	14	
Cat													
11	*6·0*	*5·6*	—	*3·8*	*3·0*	—	*2·6*	*1·4*	—	19·5	—	26	—
40	7·2	5·8	5·6	4·5	3·4	3·3	2·6	0·9	0·8	—	—	51	Foetal
	3·8	3·0	3·1	5·0	3·6	3·4	1·8	0·4	0·3	69	—	(120)	Mid
	5·2	4·5	3·8	3·4	2·2	1·6	1·0	0	0	—	—	49	Maternal
43	4·4	4·1	3·8	2·4	1·6	1·6	1·0	0·7	0·6	—	—	26	Foetal
	3·6	3·1	3·2	2·3	1·5	1·4	1·5	0·6	0·6	85	—	33	Mid
	5·3	4·5	4·4	2·8	1·8	1·5							
	4·4	3·7	3·6	2·0	1·4	1·0	1·0	0·4	0·4	—	—	30	Maternal
10	6·4	5·6	—	4·1	2·5	—	2·2	0·6	—	109	—	38	—
											Mean	36	
Ferret													
23	22	14	—	8·3	5·5	—	5·4	1·5	—	1·4	—	32	—
19	*21·4*	*19·8*	—	*8·8*	*6·2*	—	*4·7*	*1·4*	—	2·9	—	26	—
											Mean	29	
Human													
0	3·7	2·6	—	3·6	2·1	—	1·6	0·4	—	—	—	77	Maternal
28	5·7	4·5	—	4·7	2·9	—	1·7	0·4	—	—	—	61	Maternal
28	4·0	2·5	—	2·3	0·9	—	1·3	0·4	—	—	—	24	Foetal
17	4·6	4·0	—	4·5	2·8	—	—	—	—	—	—	—	Foetal
21	2·9	1·5	—	2·5	1·3	—	2·3	0·6	—	—	—	62	Foetal
				2·5	1·0								
											Mean	57	

Rodentia (placenta haemochorialis labyrinthica).

Although the placentae examined were from different stages of pregnancy, inspection of the figures shows that the glucolysis is highest in the case of the rat, the other three animals having much lower Q values. In Exp. 16 whole placentae were used; in Exp. 27 the placenta was divided horizontally into maternal and foetal halves which agreed with one another and with the slices of Exp. 33 in their metabolism.

The placentae of the four rodents hardly attacked fructose, the fructolysis being little larger than the autoglycolysis.

Carnivora (placenta endotheliochorialis).

The placenta of the cat is zonary, that of the ferret zono-discoidal [Strahl, 1906, p. 301] consisting of two discs separated on the antimesometrical side by a blood extravasation. The ferret placenta showed a very high glucolysis: fructose was also attacked but only to about one-third the extent of glucose. In the cat the glucolysis was lower, but fructose was attacked relatively to about the same extent as in the ferret.

As both maternal and foetal tissues enter into the placenta of Carnivora, we have compared the activity towards glucose and fructose at different depths through the placenta (Exps. 40 and 43, Table III). The placenta was divided horizontally into three portions, after rejecting thin layers from the outer surfaces. Slices were cut from the foetal, middle and maternal portions thus prepared and were used separately for manometric measurements. Histological examination of the portions used in Exp. 40 showed that whilst the foetal and mid parts consisted entirely of placental labyrinth, the maternal portion contained labyrinth together with the mixed maternal and foetal tissues of the "invasion-zone" [Grosser, 1909, p. 128; 1927, p. 119]. The glucolysis remains approximately constant as we pass through the placenta, although an apparently anomalous low result was obtained for the middle portion in Exp. 40. The fact that the duplicates in Exp. 43 show rather a high divergence illustrates the variable nature of the material, which makes accurate comparison difficult. The fructolysis falls off with the autoglycolysis as the maternal side is approached.

Man (placenta haemochorialis olliformis).

Full-time human placentae were obtained as already described. Slices were cut from the warm placenta, the larger white infarcts being avoided and the surface layers rejected. The Q values for full-time human placentae show a greater variation among themselves than that shown by the placental tissue of the other animals; this is no doubt due to the large amount of metabolically inert fibrinoid tissue in the human placenta. There was a relatively large autoglycolysis in the first hour, which fell to a small value in the second. In all cases the glucolysis was considerably higher than the autoglycolysis, particularly in the second hour. The figures given show no systematic difference between the metabolism of maternal and foetal sides. Both sides definitely attack fructose but to a less extent than glucose, the fructolysis falling off rather more rapidly than the glucolysis. These experiments show clearly, in contradistinction to those of Hayashi [1928] on *Brei*, that the human placenta converts glucose more readily than fructose to lactic acid, under our experimental conditions.

Embryo and foetal tissues.

Embryos were taken of a size suitable for use without cutting, and were carefully freed from membranes. Table IV shows that the rat embryo of the

Table IV. *Embryo and foetal tissues.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1	Glucolysis 2	Glucolysis 3	Fructolysis 1	Fructolysis 2	Fructolysis 3	Autoglycolysis 1	Autoglycolysis 2	Autoglycolysis 3	Embryo: Wt. Dry wt. mg.	Embryo: Age days	Relative fructolysis %
	hours			hours			hours					
						Embryo.						
Rat												
14	14	10	—	4	2·5	—	3	1·5	—	3·4	13	10
30	—	—	—	3·9	2·5	1·8	4·6	1·8	0	3·8	13	(7)
5	—	—	—	4·6	2·2	2·6	2·8	1·6	1·3	4·7	13	(6)
16	—	—	—	3·7	—	—	3·9	—	—	5·3	13–14	—
Rabbit												
26	15·9	12·9	11·2	2·8	1·4	1·3	—	—	—	12·0	14	11
Chick												
9	15·5	—	—	—	—	—	—	—	—	3·8	4	—
	12·0	—	—	—	—	—	—	—	—			
	22·5	—	—	2·3	—	—	1·7	—	—			(4)
15	—	—	—	3·5	1·2	—	2·9	1·2	—	5·2	4½	0
2	—	—	—	3·0	1·0	1·0	2·5	1·0	1·0	10·5	5	0
6	—	—	—	3·6	0·8	0·4	3·6	0·8	0·4	10·7	5	0
						Foetal tissues.						
										Moist wt. g.		
Cat-liver												
11	5·2	—	—	2·6	—	—	—	—	—	19·5	—	—
Guinea-pig-liver												
18	*3·4*	*2·6*	—	*2·6*	*1·8*	—	*2·0*	*0·6*	—	—	50	60
Mouse-liver												
42	12·5	9·6	8·9	10·4	9·2	8·8	11·6	10·2	8·3	1·0	17	—
Mouse-skin												
42	18·5	17·0	16·5	6·5	3·0	2·5	6·5	3·5	2·0	1·0	17	0
Cat-lung												
41	17	14	—	4·0	3·5	—	4·5	2·5	—	57	—	0
Cat-brain												
41	*19*	*15*	—	*2·4*	*2·0*	—	*1·8*	*1·4*	—			4

13th to 14th day and the rabbit embryo of the 14th day do not fructolyse. These embryos are in the mid-period of gestation. The chick embryo of 4 to 5 days' incubation shows a similar inability to attack fructose.

For the investigation of the later embryo, the large size necessitates the use of individual tissues. The experiments on the foetal brain, liver, lung and skin given in Table IV were designed to find out if these tissues develop any special fructolytic power in the terminal stages of gestation. The liver of the cat and guinea-pig was used in slices; that of the mouse was small enough to be used as a whole organ. In the guinea-pig there was a definite attack on glucose (contrast adult rat-liver [Rosenthal, 1930, 2]); and the fructolysis lay

between the glucolysis and autoglycolysis. In the mouse all three coincided, the autoglycolysis being extraordinarily high. The skin of the mouse embryo and slices of embryonic brain and lung of the cat, all failed to attack fructose, though their glucolysis, like that of most embryonic tissues, was high.

Chemical estimation of lactic acid formed from fructose.

The manometric measurements of the fructolysis in two representative cases, rat chorion and cat amnion, were controlled by simultaneous lactic acid determinations by Clausen's method. For this purpose the contents of the vessel were removed and acidified immediately after taking the last manometer-reading; the proteins were precipitated by tungstic acid, and sugar was removed from the filtrate by the copper-lime reagent. The filtrations were carried out through Jena glass filters. The lactic acid was determined by the method of Friedemann and Kendall [1929]; instead of aerating, however, washed carbon dioxide from a cylinder was used. Duplicate specimens of tissue were placed in the bath at the same time as those above, and were removed at the time of the first reading for determination of the preformed lactic acid. In the last experiment it was found that the extent of this correction could be greatly reduced by suspending the tissue in warm oxygenated Ringer solution for 10 minutes before the experiment. Variations in the preformed lactic acid are the principal source of error. The figures given in Table V confirm, however, that the manometric findings accurately represent lactic acid formation.

Table V.

			Lactic acid					
			Chemical estimation			Manometric		
Exp. no.	Dry wt. of tissue mg.	Time mins.	Preformed mg.	Total mg.	Total *minus* preformed	CO_2 mm.³	Lactic acid mg.	Chemical / Manometric %
Rat-yolk-sac								
3	3·7	0	0·16	—	—	—	—	—
3	2·7	180	0·11	0·81	*0·70*	195	*0·78*	90
3	3·3	180	0·14	0·95	*0·81*	228	*0·91*	89
7	3·8	0	0·14	—	—	—	—	—
7	4·3	140	0·16	0·89	*0·73*	169	*0·68*	107
39	3·2	0	0·10	—	—	—	—	—
39	5·1	0	0·18	—	—	—	—	—
39	2·3	270	0·07	0·35	*0·28*	77	*0·31*	90
39	3·6	270	0·12	0·62	*0·50*	133·5	*0·53*	94
Cat-amnion								
41	3·9	0	0·04	—	—	—	—	—
41	3·8	0	0·02	—	—	—	—	—
41	6·2	0	0·08	—	—	—	—	—
41	4·5	240	0·04	0·67	*0·63*	158	*0·63*	100
41	5·1	240	0·04	0·90	*0·86*	228	*0·91*	95

Survey of results.

Nature of the medium. It is difficult to say what medium most nearly approaches the physiological for these membranes. In the living animal the membranes may enter into relationship with foetal blood, with the foetal

fluids, or with both of these, whilst both maternal and foetal blood enter the placenta. This question is of importance since the glycolysis is to some extent dependent on the nature of the medium used. Our glucolysis values in Ringer solution for rat outer membrane fall between those obtained by Negelein [1925] in inactivated horse-serum and those of Kumanomido [1928] in rat-serum. Indeed, the latter considers that serum exerts an inhibiting action on the glucolysis of embryonic tissues. Our experiments have therefore been carried out entirely in Ringer solution.

It should be emphasised that the above results were all obtained from experiments in 0·2 % solutions of the sugars. The arbitrary concentration of 0·2 % was adopted for glucose by Warburg; this concentration does not depart widely from the physiological level, while giving a sufficient sugar reserve, and for tumour tissue there is little fall in glucolysis when the concentration is decreased from 0·2 % to 0·1 % [Warburg, Posener and Negelein, 1924]. It should be noted, however, that 0·2 % is not necessarily the optimum concentration for tissues other than tumours or for sugars other than glucose. For our experiments such a concentration may not even be physiological, since considerably higher concentrations of fructose have been reported in the foetal fluids. It is, therefore, realised that a somewhat different picture might be obtained with other fructose concentrations. Again, the experiments were carried out at the p_H of blood, from which the p_H of the various foetal fluids may differ widely [Needham, 1931, p. 1543]. However, our use of the blood-p_H and of 0·2 % sugar concentration enables our measurements in glucose to be compared directly with those of other authors, whilst the fructolysis figures allow a comparison to be made of the attack on glucose and fructose under identical conditions.

Relative fructolysis. Since the autoglycolysis is often fairly large in embryonic tissues, direct comparison of the glucolysis and fructolysis does not always give a true indication of the relative extent to which the added sugars are attacked. When sugar is present in the medium the glycolysis is not always equal to the sum of the autoglycolysis and the lactic acid formed from the added sugar. This is shown by the fact that when in the later stages of an experiment the autoglycolysis falls to a low value, glycolysis in the sugar solution frequently does not fall by the same amount and may even remain constant at its initial rate throughout (see the Tables). We can only say that a sugar is attacked, when its addition causes the autoglycolysis to be exceeded, but for the reasons given above, the excess of glycolysis over autoglycolysis does not accurately represent the amount of the added sugar converted to lactic acid. This error, however, decreases with the autoglycolysis, so that we can best estimate the lactic acid formation from added sugar in the later period of the experiments when the autoglycolysis has fallen to a low value. The relative fructolysis is then described by the percentage

$$\frac{\text{Fructolysis} - \text{Autoglycolysis}}{\text{Glucolysis} - \text{Autoglycolysis}} \times 100.$$

The values of the relative fructolysis for the second hour of the various experiments are set out in the last columns of Tables I–IV, and are represented diagrammatically for the different tissues in Figs. 1 and 2, where the

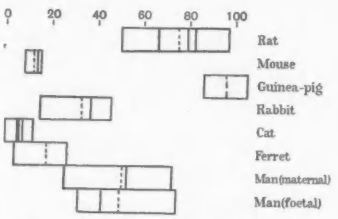

Fig. 1. Relative fructolysis of outer membrane.

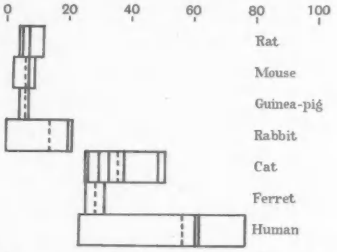

Fig. 2. Relative fructolysis of placenta.

values and their means are represented by continuous and dotted vertical lines respectively. Duplicates on the same tissue are shown as one. In a few experiments where observations over the second hour were not available, those of the second half-hour have been taken.

It will be seen that in some cases where the relative fructolysis is considerable the individual values are widely spread. Since the figures are obtained from tissues at different stages of pregnancy it might be supposed that the relative fructolysis is related to the age of the embryo. This, however, is not the case, for although the fructolysis falls with the glucolysis as pregnancy advances, the relative fructolysis shows no systematic variation. The spread of the relative fructolysis is not surprising with mixed tissues such as human and cat-placenta, but it is remarkable when it occurs in an apparently homogeneous membrane such as the rat-yolk-sac. However, even this latter membrane is by no means simple, since in the later stages it becomes villous [Robinson, 1892]. In view of the complexity of the tissues and the diverging results, it is impossible from our limited number of observations to make any far-reaching generalisations, but we wish to point out one or two inferences which are rendered probable by our findings.

Outer membrane. Considering first the yolk-sac of the rodents, it appears that the various species differ dramatically in their relative attack on fructose, rat and guinea-pig having high, and rabbit and mouse low, relative fructolysis. In contrast with this, the morphological appearances are almost identical, so much so that Grosser [1927] states: "Die Embryonalhüllen dagegen besitzen bei allen Nagern so viele auffällige gemeinsame, nur bei ihnen vorkommende Züge, dass die ganze Ordnung auf Grund der Hüllen ebensogut hätte aufgestellt werden können wie auf Grund des Zahnbaues."

Nevertheless, the previous history of the rabbit membranes is very different from that of the other three, and some difference in behaviour might be understood on this account; but the entirely different behaviour of rat and

mouse towards fructose is most surprising in view of their absolute similarity of development.

The allanto-chorion of Carnivora (cat and ferret) seems to attack fructose but little.

Placenta. In the later stages of pregnancy in the guinea-pig, rat and mouse the discoidal placenta is attached to the decidua over only a small portion of its surface. The conditions are therefore favourable for obtaining homogeneous material and consistent results. Such results indeed were obtained, the relative fructolysis being consistently low. The form of the placenta is rather different in the rabbit, and the relative fructolysis is possibly higher. It appears to be certain that the relative fructolysis is higher in the placenta of the cat and ferret than in that of the guinea-pig, rat and mouse. It is also seen from Exps. 40 and 43 (Table III) that the relative fructolysis is constant through the whole depth of the placenta, if we neglect the mid portion in Exp. 40 where the glucolysis is anomalously low.

The attack of the placenta on fructose does not depend on the extent of the erosion of the maternal uterine tissues effected by the trophoblast, for the haemochorial human placenta resembles in its relative fructolysis the syndesmochorial placenta of the carnivores rather than the haemochorial placenta of the rodents. We cannot therefore refer the fructolysis of the placenta of the carnivores to the retained maternal elements, nor can we say that the trophoblast itself behaves similarly in the placentae of the different species.

DISCUSSION.

"The fructose question," says Needham [1931, p. 1054], "is the most enigmatic aspect of embryonic carbohydrate metabolism." Whilst the appearance of fructose in pregnancy has been recorded, no way in which it may be utilised has so far been suggested. Its source is unknown, and in any case its presence in the amniotic and allantoic fluids cannot be explained until the vexed question of their origin has been settled. But the appearance of this sugar in pregnancy led us to look for a fructolysis of the foetal membranes and placenta, and this we have actually found to occur. At least one of the three tissues, outer membrane, amnion, or placenta, of the pregnant animal of every species examined (with the possible exception of the mouse in which the amnion was not investigated) was found to convert fructose into lactic acid. It would not be possible to correlate our findings with the occurrence of this sugar, which varies greatly in the different species, until complete data were available giving the amounts of fructose and glucose in the foetal fluids at different stages of pregnancy for the various species examined. The information which we have is slight, for only now are reliable methods for the determination of small quantities of fructose being developed. However, if in spite of this we examine our metabolic findings from the physiological standpoint, they are found to present several surprises. Paton, Watson and Kerr [1907]

found that among the animals we have used, fructose is a constant constituent of the allantoic and amniotic liquids of the cat and of the mixed fluids of rabbit, ferret and guinea-pig. Information on rat and mouse is lacking. We may note that in all four animals in which fructose is observed during gestation the amnion definitely fructolyses. This membrane has therefore to some extent adapted itself to the fructose-containing *milieu* which surrounds it. On the other hand, the allanto-chorion of the cat does not fructolyse, although the allantoic face of it is bathed in a medium which definitely contains fructose. Further, the placenta of the cat attacks fructose, so that chorion and placenta behave differently although they are both of trophoblastic origin.

We have shown that the whole embryo half-way through pregnancy does not utilise fructose for the formation of lactic acid, and that no special fructolytic power is developed in embryonic liver, lung or brain. Although they glucolyse readily, these tissues do not adapt themselves to the presence of the fructose in the circulating blood. It is clear also that the power to convert fructose into lactic acid is not a property always possessed by rapidly growing tissues. We have therefore neither shown a universal adaptation of embryonic tissues to the presence of fructose nor adduced a reason for its appearance during gestation. It may be that fructose is a by-product in embryonic metabolic processes of whose occurrence we are at present quite ignorant. It may be said that the fructose mystery deepens.

In seeking an explanation of the great variation in the relative attack on fructose and glucose shown by different tissues the following two main hypotheses might be advanced.

(1) Difference due to a different permeability of the tissues for fructose and glucose.

(2) Variation in the action of the "glycolytic enzyme"; or more exactly, of the system of enzymes and co-enzymes responsible for the conversion of added hexose into lactic acid. This might be due either to different modes of attack in the various tissues (different or partially different enzyme systems) or to a different reactivity towards different forms of the sugars, so that with one tissue one form (*e.g. h*-fructose) might have a higher affinity for the enzyme system than the other (*e.g. n*-fructose), whilst in another tissue the converse might hold (nomenclature of Schlubach [1932]).

Similar problems in the selective fermentation of sugar solutions by yeast have been much investigated [Willstätter and Sobotka, 1922; Hopkins, 1931; Fernbach, Schoen and Mori, 1927; Dawson, 1932]. Here most workers do not favour the permeability theory, though direct evidence is difficult to obtain. In animal tissues observations on the relative permeability of rat intestinal canal to fructose and glucose have been made by Cori [1925] who found that this tissue was freely permeable to fructose, the ratio of permeabilities of glucose and fructose being approximately 2 : 1.

It appears unlikely that a different permeability is the main factor in

deciding the extent of attack on fructose and glucose by the tissues described in the present paper. If this were so it would be necessary to assume that several morphologically and embryologically almost identical membranes (such for example as the yolk-sac of rat and mouse) which show a high and almost equal rate of attack on glucose, differ so widely in their permeability to fructose that the relative fructolysis is caused thereby to differ from nearly 0 to 100 % of the glucolysis. It is true that this possibility has not been excluded by direct experiment, and that this might be possible by a study of the oxidative reactions, as was done by Loebel [1925] for brain tissue. If those tissues which do not fructolyse should be found to oxidise fructose this would be a definite proof of its diffusion into the cells. Experiments on this point are at present in hand but some difficulty is caused since we find that the respiration of these embryonic tissues, unlike that of brain, often does not fall off markedly in the absence of added substrate, and the clear demonstration that fructose is being oxidised is thus rendered more difficult.

It should be remarked that the possibility that permeability regulates the attack on the sugar has not as yet been definitely excluded even for glucose as substrate. It has yet to be established how far the gradation in the ability of the various tissues to glucolyse [Warburg, 1927], which coincides with that in their power to oxidise, carbohydrate [Dickens and Šimer, 1930], is to be attributed to enzymic rather than diffusion differences. Nevertheless, it is usual to attribute the differing ability of various types of tissue to convert glucose into lactic acid, or to oxidise it, not to their differing permeabilities to the sugar but to the different activities of the respective enzyme systems in them. It is therefore at least as justifiable to exclude permeability questions as the deciding factor in the present problem.

It is noteworthy that in the normal tissues previously observed to convert fructose into lactic acid (muscle, liver) several apparent differences from the other body tissues are evident. These are the only tissues in which resynthesis through the Meyerhof cycle has been experimentally demonstrated. Muscle tissue attacks preferentially polysaccharides such as glycogen, starch and trihexosan, whilst the simple hexoses are less affected. The other tissues, on the other hand, react, often vigorously, with glucose and mannose converting these sugars into lactic acid, whilst fructose is hardly attacked by them. Loebel [1925] found that brain tissue could not form lactic acid from fructose though it oxidised this sugar as readily as glucose or lactic acid, thus showing that it is not impermeable to fructose. According to Ashford and Holmes [1929] the lactic acid formation in brain is of two types, the one without and the other with preliminary interaction of the carbohydrate with phosphoric acid; of these the former greatly predominates, whilst the latter, which involves glycogen, accounts for only a small fraction of the total anaerobic glycolysis. Thus in brain inability to fructolyse is associated with the absence of phosphorylation. On the other hand, Meyerhof [1930, p. 155] has shown that fructose is broken down to lactic acid rapidly by his glycolytic muscle-

extract, provided that hexokinase be added to the system. During the initial phosphorylation period the attack on fructose was twice as rapid as that on glucose. In view of these observations, Meyerhof [1930, p. 194] has suggested that the special suitability of fructose for lactic acid formation in muscle and muscle-extract is analogous to the behaviour of these two sugars in alcoholic fermentation and can be attributed to the fact that fructose is more readily phosphorylated than is glucose. "Mann kann vielleicht das abweichende Verhalten der übrigen Gewebe darauf beziehen, dass bei der Zuckerspaltung hier intermediär keine Phosphorsäureester gebildet werden. Denn ebensowenig wie Glykogen wird die Hexosephosphorsäure von diesen Geweben gespalten, mit alleiniger Ausnahme der Leber, wo ja auch Glykogen abgebaut wird und der Spaltungsmechanismus mit dem des Muskels näher übereinstimmt." To this example (liver) we may probably add a number of embryonic tissues which are also able to break down fructose to lactic acid, but possess the advantage of attacking both glucose and fructose strongly. It is probable that these tissues, showing every degree of fructolysis from nearly 0 to 100 % of the glucolysis, will provide a peculiarly suitable material for the investigation of the different mechanisms by which carbohydrate is attacked by animal tissues.

At the present time we would, therefore, merely indicate the nature of the problem. The embryonic tissues described in this paper appear to resemble muscle and liver in little but that some of them may contain fairly high concentrations of glycogen [Grosser, 1927, pp. 86, 391]. However, if Meyerhof's explanation should prove to be applicable to the different behaviour of glucose and fructose in our embryonic tissues, we should expect to find the two types of attack—with and without preliminary phosphorylation of the sugar—occurring to different extents in tissues whose behaviour towards glucose apparently gives no indication of any difference in metabolism. A striking example of this would be given by the yolk-sac of the rat and mouse, the former showing purely phosphorylation and the latter none. On the other hand, the two mechanisms might be combined in any one tissue in any proportion, resulting in an attack on fructose intermediate between the autoglycolysis and the glucolysis. This fundamental question of carbohydrate utilisation by animal tissues, which for lack of suitable material has not yet received any systematic investigation, obviously cannot be decided on the evidence at present available. In view of its importance to carbohydrate metabolism in general, we propose to continue investigations on the suitable tissues provided by the embryonic material whose behaviour has been described in the present paper.

SUMMARY.

1. A study has been made of the anaerobic conversion of fructose and glucose into lactic acid by the foetal membranes (outer membrane, amnion, yolk-sac, allantois), the placental tissues, the whole embryo and the tissues

of the later embryo (liver, lung, brain and skin). The measurements were made throughout at p_H 7·5 in bicarbonate-Ringer solution containing 0·2 % of the sugar.

2. Tissues of the rat, mouse, guinea-pig, rabbit, cat and ferret at different stages of pregnancy were examined. Whilst glucose is converted into lactic acid with great readiness by most embryonic tissues, the attack on fructose depends on both the tissue and the species, varying from zero to a value equal to that observed with the same tissue in glucose.

3. For a given tissue the figures for the relative attack on fructose and glucose may show a considerable spread. In view of this some of the results cannot be adequately summarised, but the following are the most probable conclusions.

Tissues with attack on fructose approaching or equalling that on glucose. Outer membrane of rat and guinea-pig; allantois of rabbit.

Tissues with intermediate attack on fructose. Placenta of ferret and cat; yolk-sac of cat.

Tissues with little attack on fructose. Placenta of rabbit, guinea-pig, rat and mouse; outer membrane of mouse, cat and ferret. Tissues of the embryo.

4. The amnion attacks fructose, though to a different degree in different species.

5. The placenta and peripheral membrane from the full-time human afterbirth both attack fructose, though to a less extent than glucose.

6. Neither the whole embryo at mid-term (rat, rabbit) nor the isolated tissues of the later embryo mentioned under 1 above, are able to convert fructose into lactic acid, although fructose is known to be present in the foetal blood.

7. It is concluded that in all species in which fructose is known to be present in the foetal fluids, it is a possible source of energy to at least one of the extra-embryonic foetal tissues.

8. There is no apparent correlation between the fructolytic power and the development and morphology of the tissues examined.

9. In view of Meyerhof's suggestion that ability or inability to fructolyse is connected with the existence or absence of preliminary phosphorylation, the tissues examined may well prove of value in the elucidation of the early stages of intermediate carbohydrate metabolism.

We wish to express our thanks to Prof. E. C. Dodds for his interest in these experiments, and to Dr J. H. Woodger and Dr L. W. Proger of this Hospital for their advice, and for histological examination of some of our material.

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CL. THE PREPARATION OF METHIONINE FROM CASEINOGEN.

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In the course of some work on the unidentified sulphur compounds in proteins I have found that the methods that have been used for the isolation of methionine from a protein hydrolysate are very unsatisfactory. All the methods that have been described, except that of Odake [1925] who isolated 0·0001 % of methionine from yeast, are simply modifications of Mueller's [1923] original method and involve the precipitation of the mercury compound of methionine mixed with a large amount of basic mercuric sulphate. The following yields have been obtained from caseinogen. Mueller [1923] 0·2–0·4 %, Barger and Coyne [1928] 0·1–0·3 %, du Vigneaud and Meyer [1931] 0·1–0·2 %. These low yields, and the laborious nature of the isolation, justified Barger and Weichselbaum [1931] in concluding that it was easier to make this amino-acid synthetically.

In this paper a method of preparation is described which is very much simpler than those that have already been described and which gives better yields; 1·0 % of methionine is readily obtained from caseinogen and, by working up the final mother-liquor the yield may be raised to 1·4 %.

It became obvious during the preliminary work that the precipitation of methionine as its mercury compound was unsatisfactory in the presence of large amounts of other amino-acids. An initial partial separation of the hydrolysate is therefore imperative and is most easily carried out by means of butyl alcohol. In strongly acid solution methionine is appreciably soluble in the alcohol, it may therefore be concentrated by the ordinary Dakin [1918] procedure. I have found it more convenient, however, to extract the methionine from the butyl alcohol with sodium hydroxide instead of distilling off the alcohol from the amino-acid mixture. I have carried out this double extraction in a separating funnel but it would be easier on a large scale to do it continuously. The hydrolysate should be purified somewhat, before extracting, by precipitation with lead acetate; if this precipitation is omitted a semi-solid foam is obtained when the hydrolysate is shaken with butyl alcohol. Picric acid is equally efficacious if for any reason the presence of lead in the extracted fluid is to be avoided. It does, however, precipitate a tar from the hydrolysate and for this reason I prefer lead.

Experimental.

200 g. caseinogen are hydrolysed by boiling for 14 hours with 200 cc. of water and 400 cc. of concentrated hydrochloric acid. The hydrolysate is distilled *in vacuo* till it becomes very viscous; the residue is then dissolved in 500 cc. of water. It dissolves slowly and it is generally necessary to heat the mixture. When it has cooled, 200 cc. of saturated normal lead acetate are added and the precipitate of lead chloride and tarry material is removed by filtration.

A sample of the filtrate is titrated with sodium hydroxide using bromophenol blue as an external indicator. It should be equivalent to 1–1·5 times its volume of *N* NaOH. This strongly acid solution is now extracted with 2 litres of technical butyl alcohol in a large separating funnel and the aqueous layer is run off. Sometimes it is difficult to see the interface since each layer is dark brown in colour; this difficulty can be overcome by having the apparatus in a poorly lit room and holding a powerful electric lamp close to it. The butyl alcohol in the funnel is now shaken with sufficient 10 % sodium hydroxide solution (about 250 cc.) to make it neutral. A large excess of alkali should be avoided, but it makes little difference whether the mixture is left neutral or is made definitely alkaline. The aqueous layer is separated after being left for an hour or so to settle thoroughly. It is important that the sediment which collects at the interface during the first (acid) extraction should be removed with the aqueous layer whereas that which collects during the second (alkaline) extraction should be left in the separating funnel. The acid aqueous solution from the first extraction is now returned to the funnel and extracted again with the same butyl alcohol and the butyl alcohol re-extracted with 5 % sodium hydroxide (about 300 cc. will probably be required). This alkaline aqueous extract is mixed with the first lot.

After two more double extractions as described above the greater part of the methionine, along with other amino-acids, is obtained in the alkaline solution. The same amount of butyl alcohol has been used successfully to extract as much as 800 g. of hydrolysed caseinogen, in this case the double extraction was repeated 12 times. After every second extraction 30 cc. of conc. hydrochloric acid should be added to the hydrolysate. After the last alkaline extraction a further extraction with 200 cc. of water will bring out a little more methionine.

The combined alkaline extracts are now distilled *in vacuo* till all the dissolved butyl alcohol has been removed and the volume is about 500 cc. When cold the solution is filtered and the solid is boiled with about 200 cc. of water, cooled and filtered again. The combined filtrates are brought to p_H 5 with HCl, and 200 g. of mercuric acetate dissolved in about 500 cc. of 3 % acetic acid are added. The volume should now be 1000–1500 cc.; if it is smaller than this the precipitation will be incomplete. Precipitation is complete in a minute or two and the mixture may be filtered immediately. The solid is sucked dry on the

filter, immediately ground up with 400–500 cc. of water and again filtered. The mercury precipitate should never be allowed to become parched or the subsequent extraction with baryta will be unsatisfactory. The precipitate is suspended in water and added to 600 cc. of boiling $N/3$ barium hydroxide. The mixture is heated to boiling, solid barium hydroxide being added if necessary to make the solution strongly alkaline to thymolphthalein. The mixture is filtered hot and the solid is extracted again in the same way with 700 cc. of $N/7$ barium hydroxide.

Barium is removed fairly exactly from the combined filtrates with sulphuric acid. The amount of sulphuric acid finally present should not be greater than 1 cc. of $2N$. The mixture is filtered and the filtrate distilled *in vacuo* to dryness to remove as much as possible of the acetic and hydrochloric acids. The residue is dissolved in 30 cc. of water and poured into a boiling solution of 60 g. $HgCl_2$ in 120 cc. of water. The mixture is cooled in a freezing mixture till crystals of ice separate. It is then left for an hour or two at 0° and filtered. The putty-like mass of methionine-mercury compound is kneaded with ice-cold water; this is added to the filtrate. A further quantity of less pure methionine can be obtained by adding 6–10 g. of mercuric acetate, in the form of a 30 % solution, to this filtrate and working up the precipitate as if it were a mercuric chloride precipitate.

The mercury compound is suspended in 250 cc. of water and decomposed with H_2S; the decomposition is rather slow. The filtrate from mercuric sulphide is distilled *in vacuo* nearly to dryness. Care is required at this point, for if the distillation is not carried far enough a large amount of HCl will remain with the methionine. This will necessitate the addition of a large amount of pyridine in the next stage and the yield of methionine will be small because it is appreciably soluble in alcohol in the presence of pyridine hydrochloride. If on the other hand the distillation is too violent solid methionine hydrochloride[1] will blow down the condenser as a fine dust. I have found it safest to connect the distillation flask to the condenser by means of a piece of pressure tubing, this can be clamped off at the critical moment. 30 cc. of alcohol are used to dissolve the solid methionine hydrochloride; if necessary the alcoholic solution is filtered and the flask washed out with a further 15 cc. of alcohol.

Pyridine is now added until a drop of the solution on a tile is alkaline to bromophenyl blue; 2 cc. more of pyridine are added and the mixture cooled for some hours in a freezing mixture. It is filtered and the solid washed with a little alcohol.

Yield, 1·8 g. of methionine of 95 % purity.

When measured in 3 % aqueous solution $[\alpha]^{21°}_{5461} = -7{\cdot}9°$.

From the mercuric acetate precipitate a further 0·8 g. is obtained. This is only 75 % pure but may be purified by reprecipitation with mercuric chloride.

[1] Methionine, like ethylcysteine, forms a hydrochloride, which crystallises from strong hydrochloric acid in clusters of long needles and does not lose hydrochloric acid when dried *in vacuo*. It melts sharply at 174° (uncorr.) but solidifies again, and on further heating melts at 210–223°. Found: N, 7·2; S, 17·22; Cl, 19·4. Calculated: N, 7·54; S, 17·25; Cl, 19·12 %.

In each case the alcoholic mother-liquor contains sulphur to the extent of about 1·5 mg./cc. In this case there is therefore about 150 mg. of sulphur still present in the mother-liquor. The greater part of this is in the form of methionine and I have isolated it in about 70 % yield (reckoned on the sulphur content) by making the combined mother-liquors from several preparations alkaline (with barium hydroxide) and distilling *in vacuo* to get rid of pyridine, followed by acidification (with sulphuric acid) and precipitation with mercuric chloride.

The yield of methionine isolated in this way is therefore 1·4 %. This is considerably less than the actual methionine content of caseinogen, for more may be obtained by more prolonged extraction with butyl alcohol and also by precipitating with a larger amount of mercuric acetate. Both of these procedures, however, involve more labour than the extra yield is worth.

Methionine may also be isolated by following the method already described but with the omission of the butyl alcohol extractions. In this case the yield is much smaller and is very variable; the method then differs from those that have already been described by other workers only in the shortening of some of the processes and gives a very similar yield, *i.e.* 0·25–0·7 %.

Discussion.

The commercial caseinogen which has been used in this work contains 0·6 % of sulphur (uncorrected for moisture or ash) and from it 1·4 % of methionine has been isolated; this, since methionine contains 21·5 % of sulphur, corresponds to 0·3 % of sulphur in the protein. It is clear therefore that methionine is the principal sulphur-containing constituent of caseinogen.

The yield of methionine is not quite high enough definitely to rule out the possibility of a third sulphur-containing amino-acid. Most workers have put the cystine content of caseinogen at 0·3 % [*e.g.* Sullivan, 1929]; on this basis there is a considerable amount of sulphur in caseinogen still unaccounted for but it is probable that part of this is methionine. Baernstein [1930], by oxidation with iodine, finds 0·94 % of cystine in caseinogen; on this basis there can be no other sulphur-containing constituent. Sherman and Woods [1925], by feeding experiments, found a still higher value, 1·2–2·5 %. This value is clearly impossible and it seems from the recent work of Jackson and Block [1931] and of Weichselbaum, Weichselbaum and Stewart [1932] that their method of estimation does not distinguish between methionine and cystine. Steudel and Schumann [1929] found, by the Folin-Looney [1922] technique, 2·6 % of "cystine" in caseinogen which had been treated with nitrous acid. This value, like that of Sherman and Woods, appears to be the sum of the cystine and the methionine contents of the protein, but the nature of the reaction involved is obscure; free methionine, on treatment with nitrous acid, does not give a product that reacts with the Folin-Looney reagent.

SUMMARY.

The preparation of methionine from the butyl alcohol extract of an acid hydrolysate of caseinogen is described.

The yield is 1·4 %.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

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CLI. THE DETERMINATION OF CELLULOSE IN SOIL[1].

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Introductory.

During the course of an investigation into the decomposition of green manures in soil it was necessary to determine the amount of cellulose that underwent changes at the different stages of fermentation. Charpentier [1920] has shown that when cellulose, in the form of filter-paper, is mixed with soil it can be recovered quantitatively by extraction with Schweitzer's reagent, an observation later confirmed by Barthel and Bengtsson [1924]. This method, however, cannot be applied directly to soils mixed with plant materials, as the plant tissues contain, amongst other constituents, hemicelluloses and lignin which interfere with the extraction of cellulose with Schweitzer's reagent. The hemicelluloses are to a certain extent soluble in Schweitzer's reagent and are precipitated with alcohol. Lignin on the other hand acts as a barrier to the complete dissolution of cellulose in Schweitzer's reagent.

Waksman and Tenney [1927] suggested treating the material with a 5 % solution of NaOH for 30 minutes at 15 lbs. pressure in order to remove the hemicelluloses and lignin before extracting the cellulose with Schweitzer's reagent. Solutions of sodium hydroxide of strength greater than 1–2 % are known to attack cellulose, so that any treatment in which the plant material is exposed to the action of alkali of more than 1–2 % strength at high temperatures is bound to give a low yield of cellulose. Bengtsson [1924] proposed treating the soil mixed with plant materials with a solution of sodium bisulphite in hydrochloric acid at a temperature of 100° for 72 hours and then extracting cellulose with Schweitzer's reagent. Apart from the fact that the sulphite process of separating cellulose gives lower yields than the Cross and Bevan method, the period of digestion required for the preliminary treatment of the material makes the process too long.

With a view to the elimination of these disadvantages an attempt was made to find a suitable method for the estimation of cellulose in plant materials mixed with soil.

[1] This paper is an abridged form of part of a thesis approved for the degree of Ph.D. in the University of London.

Experimental.

Some preliminary experiments were carried out on a sample of oat straw whose cellulose content was known. It contained 39·3 % cellulose by Jenkins's method [1930] which gives as good results as the Cross and Bevan method. In all subsequent experiments this figure was taken as the standard with which the results of the various methods tried were compared.

An attempt was first made to see if Schweitzer's reagent could extract all the cellulose from the straw after the hemicelluloses were removed as suggested by Waksman [1927]. One g. portions of straw were treated with 100 cc. of 1 % NaOH and 1 % H_2SO_4 at boiling temperature for half an hour with each reagent. The residue, after thorough washing and drying, was shaken with 100 cc. of Schweitzer's reagent for one hour[1] and the cellulose estimated according to Charpentier's process. The residue was re-extracted twice with Schweitzer's reagent and cellulose precipitated each time as before. The results (Table I) show that even by extracting the material three times, a part of the

Table I.

	1st Extraction	2nd Extraction	3rd Extraction	Total
Cellulose on dry matter, %	29·5 27·6	2·94 3·05	2·29 1·66	34·37 32·31

cellulose only could be recovered. There is a progressive dissolution of cellulose, its complete dissolution being hindered by lignin with which it is supposed to be encrusted.

The use of chlorine gas for removing lignin, apart from its other drawbacks, is ruled out where the material under treatment is mixed with soil. The sulphite process of Bengtsson, already referred to, was tried on one g. portions of the oat straw (Table II).

Table II.

	1st Extraction	2nd Extraction	Total
Cellulose % on dry matter	36·7 37·5	0·86 0·50	37·56 38·00

Though the removal of lignin has increased the amount of cellulose that could be extracted with Schweitzer's reagent, yet the yield is not up to the standard. This suggests that a part of the cellulose has been attacked during the preliminary digestion with the bisulphite. It is clear that it is necessary to remove lignin from the plant tissues before the cellulose can be completely dissolved by the Schweitzer's reagent, and yet the reagent employed must be such as to have a minimum effect on the cellulose. Jenkins's method of chlorinating lignin by means of sodium hypochlorite in an alkaline solution suggested itself and was next tried. One g. portions of the straw were subjected to the

[1] In some preliminary experiments it was found that shaking for one hour extracted as much cellulose as shaking for 6 or 24 hours.

dilute alkali and acid treatments as before and the residues therefrom were treated twice with sodium hypochlorite as described by Jenkins. The excess of hypochlorite was removed and the residues, after drying, were extracted with 100 cc. of Schweitzer's reagent and cellulose was estimated as before (Table III).

Table III.

No. of determination	Cellulose % on dry matter
1	40·15
2	39·96
3	40·05
4	39·07
5	40·82
Mean value	40·01 ± 0·28

The Schweitzer's reagent is now able to extract the whole of the cellulose at one extraction and the results agree well with the standard.

The straw was now mixed with a light sandy soil, and the mixture subjected to the same treatments as with straw alone. 10 g. of the soil mixed with 1 g. of the straw were used for analysis (Table IV).

Table IV.

		Cellulose in mixture of soil + straw	
No. of determination	Cellulose in soil %	Amount obtained	Amount calculated
1	0·0627	0·3955	
2	0·0546	0·3987	
3	0·0627	0·3915	
4	—	0·3858	
5	—	0·3851	
Mean value	0·0600 ± 0·0027	0·3913 ± 0·0027	0·4060

The results show that almost the whole of the cellulose added to the soil in the form of straw can be recovered by the method of analysis employed. A part of the cellulose, 0·0147 g. could not be recovered from the mixture. This loss of cellulose which was also observed by Charpentier and others is attributed by them to adsorption by the soil. Whatever the explanation be, it seems certain that a part of the added cellulose, in whatever form it is added, is always retained by the soil and is not recovered by the method of analysis used.

The method was then applied to different plant materials and to mixtures of these plant materials with soil. In all cases 10 g. of the soil together with 1 g. of the plant material were taken for analysis. Table V shows the results of analysis of plant materials alone. In Table VI the figures are given for the same plant materials mixed with the soil.

The results show that the cellulose added to the soil in the form of plant materials of widely different nature and age can be recovered almost quantitatively.

Table V.

No. of determinations	Cellulose on 100 g. dry matter: Young tares	Young tares by Jenkins's method	Young mustard	Sugar beet tops	Sugar beet tops by Jenkins's method	Mature mustard
1	9·83	9·62	9·42	7·18	7·87	22·90
2	10·48	10·11	8·60	8·12	7·79	23·75
3	9·21	—	—	8·09	—	—
4	9·52	—	—	7·61	—	—
5	9·83	—	—	6·53	—	—
6	9·28	—	—	6·79	—	—
Mean value	9·69	9·87	9·01	7·39	7·83	23·32
Standard error	0·18	—	0·51	0·26	—	0·42

Table VI.

	Cellulose in 10 g. soil + 1 g. plant material, expressed on dry matter: Cellulose g.	Average value	Amount calculated	Difference
Young tares +soil	0·0926 0·1015	0·0970	0·1028	0·0058
Young mustard +soil	0·0968 0·0833	0·0901	0·0960	0·0059
Sugar beet tops +soil	0·0731 0·0731 0·0745 0·0731	0·0735	0·0798	0·0063
Mature mustard +soil	0·2375 0·2350	0·2362	0·2391	0·0029

Description of method.

Ten g. of soil are heated in a beaker with 100 cc. of 1 % NaOH and allowed to boil for 20–30 minutes, maintaining a constant volume during heating. The soil is allowed to settle; this may be hastened by making the suspension slightly acid. The upper liquid is filtered off and the residue washed twice with hot water. The material on the filter is transferred to the beaker and the soil heated with 100 cc. of 1 % hydrochloric acid for about 20 minutes, maintaining a constant volume of liquid. After settling, the upper liquid is filtered off and the residue washed free of acid with hot water. The material on the filter-paper is returned to the beaker, 5 cc. of sodium hypochlorite, having 15 % available chlorine, are added and the volume is made up to 100 cc. The liquid must remain alkaline. It is allowed to stand for half an hour in the cold with three or four shakings during the interval. The upper liquid is filtered and the material on the filter returned to the beaker, another 5 cc. of the sodium hypochlorite solution added and the solution made up to 100 cc. and allowed to stand for half an hour with shakings as before. As much as possible of the upper liquid is filtered and to the residue in the beaker a dilute solution of H_2O_2 (10 cc. of 20 vol. H_2O_2 made up to 100 cc.) is gently added till effervescence

ceases, excess being avoided. The suspension is allowed to settle, being hastened with HCl if necessary, and the clear liquid is filtered through the same filter-paper. The residue in the beaker is transferred to an evaporating dish and washed thoroughly free of acid with hot water by decantation. The material on the filter-paper is transferred to the dish and the residue evaporated to dryness on a water-bath.

When the residue is completely dry, it is transferred to a suitable bottle for extraction with Schweitzer's reagent. 100 cc. of Schweitzer's reagent are added and the material shaken for one hour. It is then allowed to settle, preferably overnight. More than 50 cc. are filtered, a suction pump being used if necessary. To 50 cc. of the filtrate in a 400 cc. beaker 200 cc. of 80 % alcohol are added, and the precipitate is allowed to settle overnight. It is then filtered by decantation through a suitable filter aided by suction, Jena glass filter-crucible No. 1G4 being very convenient. After the upper liquid has all been filtered, 50 cc. of a mixture of alcohol and HCl (40 cc. of 80 % alcohol *plus* 10 cc. HCl) are added to the precipitate; the mixture is shaken till all the copper hydroxide is dissolved and is then allowed to stand for about an hour. The upper liquid is filtered through the same filter, the precipitate washed free of copper with hot water, and transferred to the crucible, washed further with hot water, then with alcohol and ether in turn as directed by Charpentier. The crucible is dried in an oven for about one hour; the cellulose is transferred to a weighed crucible, weighed, ignited and again weighed. The loss of weight on ignition multiplied by 2 represents the amount of cellulose in 10 g. of the soil.

Summary.

1. A method is described for the determination of cellulose in soil mixed with plant materials. It is essentially a combination of the Jenkins and Charpentier methods. The soil is treated with hot dilute alkali and acid and then with a solution of sodium hypochlorite in the cold. Cellulose is then extracted from the residue with Schweitzer's reagent, precipitated with alcohol and determined by loss of weight on ignition.

2. The results obtained by this method on plant materials alone agree very closely with those given by the hypochlorite method of Jenkins.

3. It is shown that it is necessary to remove the lignin from the plant tissues, otherwise it is not possible to obtain a quantitative yield of cellulose by extraction with Schweitzer's reagent.

4. The method has proved its value in recovering almost the whole of the cellulose added, when different plant materials are mixed with soil. A part of the cellulose is, however, retained by the soil and is not extracted by the Schweitzer's reagent.

5. No attempt has been made to apply the method to different types of soil.

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CLII. OXIDATIONS CATALYSED BY HORSE-RADISH- AND MILK-PEROXIDASES.

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In a previous paper [Elliott, 1932] a method was described for discovering whether any substance was oxidisable by H_2O_2 under the influence of peroxidase catalysis, and it was shown that the range of substances whose oxidation was catalysed by milk-peroxidase was very limited. Very dilute H_2O_2 with the enzyme had no effect on the various fatty acids, fats, sugars, most amino-acids, and other possible metabolites tried. Other than those compounds used in ordinary tests for peroxidase, the only substances found to be oxidised were tryptophan and tyrosine, and the oxidation of nitrite, previously observed by others, was fully confirmed.

The peroxidase from horseradish is one of the most intensively studied of enzymes and yet very little has up to now been known of its activity as a catalyst of biological oxidations. This fact and the interest of comparing the activity of an enzyme of plant origin with the corresponding enzyme from an animal source, led me to apply to horseradish-peroxidase preparations the same method as utilised with the milk-enzyme. The results obtained indicated a few more experiments to be made with the milk-enzyme.

Preparation of horseradish-peroxidase.

For this work it was necessary to have an enzyme preparation free from reducing substances and catalase, but a highly concentrated enzyme was not required. The "crude" product of Willstätter and Stoll [1918] would probably have been suitable but its preparation is laborious and occupies several weeks. The following entirely different method was found to be very satisfactory.

1470 g. of horseradish were washed and without further treatment chopped up and passed through an ordinary meat-mincer. To the mince 2 litres[2] of distilled water were added and the mixture stirred frequently during 2 hours. The liquid was squeezed through a cloth, and another extraction was made with 2 litres of water in the same way. A third extraction was done with 2 litres allowing the mixture to stand overnight before straining off the liquid. Qualitative tests showed that the third extract was considerably less active

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[2] It has since been found better to make the 3 or 4 extractions with only one litre each time.

than the first two, and though a large amount of enzyme remained in the solid it was not economical to extract it further.

The combined extracts, 6400 cc. including juice, were centrifuged or filtered through fluted filter-papers, and the translucent liquid was then fully saturated with ammonium sulphate. This precipitated the enzyme completely, together with a considerable amount of insoluble impurity. The precipitate was separated by centrifuging or on Büchner funnels, and lixiviated with a little water (250 cc. in this example, but preferably less). The turbid solution was dialysed through animal (or parchment) membrane for 3 days with frequent changes of outside water, until salt-free. The fluid, which had doubled in volume during the dialysis, was filtered clear and twice its volume, 1000 cc., of denatured[1] alcohol was added. The precipitate which formed was collected by centrifuging and dried in a vacuum desiccator. To prevent the alcohol paste splashing during the drying, it was covered by a slanting large perforated disc.

470 mg. of dried and ground up powder were obtained, having a purpurogallin number of 25·3.

To the alcoholic liquid a further 2 volumes, 1000 cc., of alcohol was added and the precipitate collected and dried as before.

125 mg. of a powder of purpurogallin number 212 were obtained.

In another preparation carried out as above the whole four volumes of alcohol were added together and the product obtained weighed 410 mg. with purpurogallin number 95·2.

The material obtained by this method is not easily soluble, and for estimation and other purposes it is necessary to grind it up carefully to a paste with very little water before diluting.

The whole preparation, including the period of dialysis can easily be finished in one week.

Oxidations by peroxidase and H_2O_2.

The method used was that described in the previous paper (pp. 16 and 19), the only change being that the experiments were made at 20° instead of at 37°.

Nitrite. It was previously shown that nitrite is quantitatively oxidised by H_2O_2 under the influence of milk-peroxidase catalysis; this oxidation was used as a test of the method, and hence nitrite was the first substance tried with the plant-enzyme. The remarkable result was obtained that horseradish-peroxidase is completely unable to catalyse the oxidation of nitrite.

Experiments carried out exactly as with milk-peroxidase showed no H_2O_2 usage at all, and, using very small amounts of nitrite, the Griess-Ilosvay test at the end of experiments showed no diminution in nitrite whatever. Experiments were made with the same result at room temperature and at 37°, at various hydrogen ion concentrations between p_H 5·7 and 7, using more H_2O_2,

[1] EtOH 96 %, MeOH 2·5 %.

or increasing the time allowed for the reaction, and with various preparations of the enzyme including a Willstätter adsorption product kindly given me by Dr H. Sutter.

The following is the summary of one experiment.

Flasks A and B each contained 20 cc. of a solution of 55 mg. of an enzyme preparation of P.Z.[1] 54, in phosphate buffer at p_H 6. A, contained 3 cc. of 3 mg./cc. sodium nitrite solution; B, 3 cc. of water. During 45 minutes at 20°, 10 cc. of 0·02 % H_2O_2 were run into each flask; after a further 15 minutes small samples tested with *p*-phenylenediamine showed by immediate coloration that both active enzyme and H_2O_2 were still present. On estimating the H_2O_2 remaining by the MnO_2 method in neutral solution, 0·64 cc. and 0·68 cc. of O_2 were liberated from the contents of flasks A and B respectively. No usage of H_2O_2 had therefore taken place. A similar experiment using only 0·5 cc. of nitrite solution, the H_2O_2 therefore being in excess, showed the same intensity of colour with the Griess-Ilosvay reagent after the experiment as a standard solution of the nitrite of the same dilution.

Qualitative tests showed that the enzyme was still active at the end of the experiments. In order to make sure that no inactivation at all was caused by the nitrous acid at the slightly acid reaction, the following experiment was made. To 5 cc. of a solution of 5 mg. of peroxidase of P.Z. 54 in phosphate buffer p_H 5·5, 2 cc. of nitrite solution 3 mg./cc. were added and the mixture kept for 1½ hours. On diluting and carrying out an estimation, the enzyme was found to have lost none of its activity.

Thurlow [1925] was also unable to show the oxidation of nitrite by plant-peroxidase but believed this to be due to reducing substances in her crude preparation reacting with the nitrate formed. My method excludes this possibility since it shows that not only does nitrite not disappear, but H_2O_2 is not used up.

The slight colour mentioned in the last paper as developing when H_2O_2 was added to nitrite and milk-peroxidase solution was again observed with the plant-peroxidase. It is not formed in the absence of nitrite or of enzyme and is therefore difficult to account for unless traces of nitrite are oxidised and the trace of nascent nitrate formed gives a xanthoproteic reaction. The colour deepens with ammonia.

It is necessary here to correct an error in my previous paper. I have found that in acid medium nitrous acid reduces H_2O_2 quantitatively, so that H_2O_2 cannot be estimated in acidified solutions containing nitrite. In Tables III, IV, V and VI of that paper the fact that no O_2 was liberated by MnO_2 in acidified solution in the presence of excess of nitrite is therefore meaningless. But in all those tables the qualitative tests recorded show that the H_2O_2 had all been used up before adding acid. The estimations in Table II showing the oxidation of nitrite were done in neutral solution, as also were the experiments on nitrite recorded here above. These estimations and all results and conclusions are therefore perfectly valid. Careful controls have shown that nitrite in neutral solution and in buffer as acid as p_H 5·5, does not affect the H_2O_2 estimation by MnO_2.

[1] P.Z. (=Purpurogallin Zahl) is the usual abbreviation for Purpurogallin Number as defined by Willstätter and Stoll [1918].

Possible metabolites. Besides nitrite the two other substances found to be oxidised under the influence of milk-peroxidase catalysis were tryptophan and tyrosine. With the plant-enzyme tyrosine was found to be oxidised, but no sign of oxidation of tryptophan could be observed; we have here therefore another remarkable difference between the two enzymes. The following compounds of different types were tried with the plant-peroxidase preparation and found, as with milk-peroxidase, not to be oxidisable; *viz.* formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

It was previously shown that dihydroxyacetone and phenylglyoxal are oxidisable by the very dilute H_2O_2 without peroxidase catalysis; the same can now be said of formaldehyde.

Tables I–V give the experimental details of some of the above results.

The amount of enzyme used in all experiments after Table I is about half of what was used in the previous paper, the smaller amount being used to economise with the enzyme preparations and to diminish the slight destruction of H_2O_2 by catalase. The experiments were all done under roughly the same conditions of H_2O_2 concentration, p_H and temperature as in the ordinary purpurogallin method of estimation. Calculating, therefore, from the rate of H_2O_2 usage in the purpurogallin method, this smaller amount of enzyme would be 2250 times enough to use up all the H_2O_2 in the time of the experiments if the enzyme worked equally rapidly with other reducing substrates. Insufficiency of enzyme, therefore, cannot account for the negative results obtained.

Table I.

Each flask contained 1·15 P.E.* of enzyme (*i.e.* 12 mg. of P.Z. 95) in 20 cc. phosphate buffer p_H 6·4, and the neutralised substrate in 5 cc. H_2O. 10 cc. of 0·02 % H_2O_2 were added over 45 minutes. Total time 1 hour before removing 1 cc. sample and adding acid. Temperature 20°.

		p-Phenylene-diamine 50 mg.	Tyrosine 50 mg.	Trypto-phan 50 mg.	Histidine-HCl 50 mg.	Controls	
						Water	Water
Colour formed		Blue	Yellow	–	–	–	H_2O_2 all added together and acid at once
Sample	Colour with *p*-phenylene-diamine	.	–	+ +	+ +	+ +	.
	Do. *plus* H_2O_2	.	+ + + +	+ + + +	+ + + +	+ + + +	.
H_2O_2 remaining at end. (cc. O_2 evolved by MnO_2)		0·06	0·0	0·67	0·66	0·57	1·61

* P.E. (= Purpurogallin Einheit) is the usual abbrevation for Purpurogallin Unit as defined by Willstätter and Pollinger [1923].

p-Phenylenediamine and tyrosine use up H_2O_2 and are therefore oxidised. Tryptophan and histidine are not oxidised. The enzyme is always still active at the end of the experiment.

Table II.

As for Table I except that each flask contained only 0·64 P.E. of enzyme, *i.e.* 6·7 mg. of P.Z. 95.

		Glycine 25 mg.	Glutamic acid 50 mg.	Acetic acid 25 mg.	Sodium lactate 50 mg.	Alcohol 25 mg.	Controls: Water	Controls: All H_2O_2 added together and acid at once
Sample	Colour with *p*-phenylenediamine	+ +	+ +	+ +	+ +	+ +	+ +	·
	Do. *plus* H_2O_2	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	·
cc. O_2 evolved by MnO_2 at end		0·87	0·82	0·87	0·86	0·84	0·87	1·45

None of the above compounds is oxidised. The enzyme is active at the end of all experiments.

Table III.

0·63 P.E. of enzyme in each flask.

		Formic acid 25 mg.	Glucose 50 mg.	Formaldehyde 30 mg.	Glycerol 40 mg.	Acetaldehyde 40 mg.	Water
Sample	Colour with *p*-phenylenediamine	+ +	+ +	(+)	+ +	+ +	+ +
	Do. *plus* H_2O_2	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
cc. O_2 evolved by MnO_2		0·92	0·80	0·14	0·86	1·16	0·85

Formic acid, glucose, glycerol, and acetaldehyde are not oxidised. The curious effect of rather greater liberation of O_2 in the presence of acetaldehyde than in its absence occurred also with milk-peroxidase. I have shown by experiments that acetaldehyde in acid solution yields no gas with MnO_2 nor is extra gas yielded on decomposing H_2O_2 when acetaldehyde is present. One can only assume that the aldehyde protects the H_2O_2 to some extent from the slight decomposition by catalase during the experiment.

Formaldehyde used up the hydrogen peroxide but separate experiments showed that this occurred to the same extent in the absence of any enzyme.

Table IV.

		Brucine 60 mg.	Fructose 90 mg.	Oleic acid 50 mg. in neutral emulsion	Tannin 100 mg. Dark green colour formed	Water
Sample	Colour with *p*-phenylenediamine	+ +	+ +	+ +	–	+ +
	Do. *plus* H_2O_2	+ + +	+ + + +	+ + + +	+ +	+ + + +
cc. O_2 evolved by MnO_2 at end		0·66	0·63	0·62	0·0 In neutral solution	0·56

Table V.

		Phenyl-alanine 50 mg.	Tyrosine 25 mg. powdered	Catechol 25 mg.	Gallic acid 40 mg.	Water
Colour formed		–	Yellow	Deep yellow	Dark greenish brown	–
Sample	Colour with *p*-phenylenediamine	+ +	–	–	–	+ +
	Do. *plus* H_2O_2	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
cc. O_2 evolved by MnO_2 at end		0·97	0·0	0·0	0·15	1·01

Phenylalanine is not oxidised while tyrosine, catechol, and gallic acid are.

Phenols. Tyrosine is acted upon by H_2O_2 and peroxidase and it has long been known that various other phenols are oxidised, the reactions with them having been used as tests for the enzyme [see Raper, 1928]. Experiments were made with a representative series of phenolic substances with the result that one may say that all substances containing a phenolic group are oxidisable by H_2O_2 under the influence of catalysis by both horseradish- and milk-peroxidase. Only one exception has been found, namely resorcinol, which, though oxidised by the plant-enzyme, completely inhibits the activity of the milk-enzyme; it is not oxidised itself and in small amount prevents the oxidation of iodide, benzidine, *p*-phenylenediamine, *etc.*

Table VI gives a summary of the observations on phenols. In this table under "qualitative" are given the observations in simple test-tube experi-

Table VI.

	Plant-peroxidase		Milk-peroxidase	
	Qualitative	Quantitative	Qualitative	Quantitative
Phenol	Yellow		Yellow	
o-Cresol	Greenish-yellow + ppt.		Greenish-yellow	
p-Cresol	White ppt.		–	+
m-Cresol	White ppt.	+ Faint yellow	–	+ Faint yellow
Vanillin	White ppt.	+ Faint yellow	–	+ Yellow
Tyrosine	–	+ Yellow	–	+ Yellow
Tyramine	–	+	–	+
Catechol	Yellow	+ Deep yellow	Yellow	
Resorcinol	Brownish-yellow		Inhibition	
Quinol	Pale yellow		Pale yellow	
Protocatechuic acid	Yellow		Yellow	
3 : 4-Dihydroxy-phenylalanine	Red-brown		Red-brown	
Adrenaline	Red		Red	
Pyrogallol	Yellow		Yellow	
Gallic acid	Green-brown	+ Colour	Green-brown	
Tannic acid	Dirty yellow	+ Colour	Dirty yellow. Enzyme largely precipitated with protein	+ Colour
α-Naphthol	Magenta + ppt.	+ Colour	Magenta	+ Colour
β-Naphthol	White ppt.		–	+ Faint yellow

– signifies "nothing easily observable." + signifies oxidised.

ments. A number of the substances, especially with the milk-enzyme, showed no marked colour or precipitation reaction; these were therefore tested by the quantitative method of estimating H_2O_2 usage, and a number of the others were confirmed by this method. The results are shown under the heading "quantitative."

No attempt has been made to discover the products of reaction.

Tables VII–VIII give the details of some of these "quantitative" experiments.

Table VII.

Horseradish-peroxidase. 0·66 P.E. in each flask.

		Tyramine 40 mg.	Gallic acid 40 mg.	α-Naphthol 40 mg.	Vanillin 40 mg.	*m*-Cresol 40 mg.	Water
Colour formed		?Scarcely detectable	Violet going at once green-brown	Light yellow with white ppt.	Faint yellow white ppt.	Light yellow milky white ppt.	–
Sample	Colour with *p*-phenylene-diamine	–	–	–	–	–	+ +
	Do. *plus* H_2O_2	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +
cc. O evolved by MnO_2 at end		0·0	0·15 In neutral solution	0·0	0·0	0·0	0·81

Table VIII.

Milk-peroxidase. 0·7 P.E. in each flask.

		Tyr-amine 40 mg.	α-Naph-thol 40 mg.	β-Naph-thol 40 mg.	Vanillin 50 mg.	*m*-Cresol 50 mg.	*p*-Cresol 40 mg.	Tannic acid 40 mg.	Water
Colour formed		Scarcely detect-able	Ma-genta	Faint yellow	Faint yellow	Faint yellow	White opal-escence	Yellow-brown	.
Sample	Colour with *p*-phenylene-diamine	–	–	–	–	–	–	–	+ + +
	Do. *plus* H_2O_2	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	(+)*	+ + +
cc. O_2 evolved by MnO_2 at end		0·0	0·0	0·0	0·0	0·0	0·0	0·39	0·96

* Considerable precipitation of enzyme had taken place.

Experiment showed that none of these substances took up H_2O_2 in the absence of enzyme; all of them are therefore oxidised under the influence of catalysis by the enzyme.

The determination of unused H_2O_2 in the experiments with tannin and gallic acid by the MnO_2 method had to be made in neutral solution since it was found that acid solutions of these substances, and of pyrogallol, evolve an uncertain amount of gas (oxygen?) when shaken with MnO_2 in the absence of any H_2O_2.

Dilute iron solutions (0·1 mg. of $FeSO_4$, $7H_2O$ or $FeCl_3$) with dilute H_2O_2 oxidised some of the above phenols slowly, but in no case was the reaction anything like as strong as with the enzyme solutions.

Pyrrole was tried (50 mg. and 15 mg. in the usual volume) and found not to be oxidised with milk- or horseradish-peroxidases. Its presence causes probably complete inhibition of the milk-enzyme and very great inhibition of the plant-enzyme. With pyridine also, some degree of inhibition of the activity of both enzymes was observed.

Discussion.

The following is a list of the substances shown, in this and my last paper and by previous workers, to be oxidised by H_2O_2 under the catalysis of the peroxidases of milk and horseradish. The numerous compounds of various types tried and found not to be oxidised by the system are listed elsewhere.

Milk-peroxidase.

Iodide, nitrite, tryptophan.
Diamines such as *o*- and *p*-phenylenediamine, benzidine, *etc.*
Guaiacum and all phenolic substances (except resorcinol which inhibits the enzyme).

Horseradish-peroxidase.

Iodide.
Diamines such as benzidine, phenylenediamines, leuco-malachite green, *etc.*
Guaiacum and all phenolic substances.

Studies of these two very similar enzymes have brought to light a number of interesting differences between them which should have some bearing on theories of the mechanism of their actions. The difference in p_H range of activity mentioned in the last paper is probably a function of the accompanying colloidal material in the preparation; the inhibition by resorcinol of the milk-enzyme, but not of the plant preparation, may possibly also have something to do with the colloidal states of the two preparations. But the oxidations of nitrite and tryptophan by the one and not by the other seem to be real differences between the active systems. Also a glance at Table VI suggests that the products of oxidation are not always the same in the two cases. As to the specificity towards the oxidising substrate, Wieland and Sutter [1930] have studied the activation of various organic peroxides by horseradish-peroxidase and it would be interesting to see their work repeated with the milk-enzyme. Dixon [1929] has stated that milk- but not horseradish-peroxidase will use persulphate as oxidising substrate.

The theory of the mechanism of peroxidase reaction put forward by Mann [1931] could perhaps explain these differences by asssuming differences in the specificity of the linkages combining the two substrates to the enzyme.

The present extension of my last paper has not thrown any further light on the place of peroxidase as a constituent of the respiratory mechanism. If a function is to be found for peroxidase it may be, as suggested before, that in its normal environment the enzyme is provided with substrates "activated" by other catalysts; its ready ability to form coloured oxidation products may be of importance, though the non-oxidation of, and inhibition by, pyrrole does not seem to support this in the case of animal tissues.

In view of the general reaction demonstrated with phenols, some revision of the present ideas concerning the interrelation of oxidases and peroxidases in plant tissues may be necessary.

Summary.

1. The preparation of horseradish-peroxidase in fairly concentrated form is described. The method consists simply in extraction of the minced root with water, precipitation of the enzyme by saturation with ammonium sulphate, purification of the precipitate by dialysis and filtration, and finally fractional precipitation by alcohol.

2. Using the method described by Elliott [1932] it was shown that nitrite and tryptophan are not oxidisable by H_2O_2 with the plant-enzyme, which is therefore different from the milk-peroxidase.

3. The following substances have also been tried with the horseradish-peroxidase and found not to be oxidised: formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

4. Formaldehyde, like dihydroxyacetone and phenylglyoxal as shown previously, is oxidised by very dilute H_2O_2 without enzyme.

5. Positive results with a representative series of phenols allow the conclusion to be drawn that all phenolic substances are oxidised by H_2O_2 with both horseradish- and milk-peroxidases.

6. To the above generalisation one exception was found. Resorcinol, though oxidised with the plant-enzyme, is not oxidised by milk-peroxidase the activity of which it inhibits.

7. Pyrrole is not oxidised with either enzyme and also has an inhibitory action which appears more complete with the milk-enzyme. Pyridine also has some inhibitory effect on the action of both enzymes.

It is a pleasure for me here to record my gratitude to Geheimrat H. Wieland for allowing me to work in his department, and for his kind interest in this work. I am also grateful to Sir F. G. Hopkins for his encouragement.

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CLIII. A POLYSACCHARIDE FROM THE POLLEN OF TIMOTHY GRASS (*PHLEUM PRATENSE*).

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Recent investigations of the relationship of various constituents of pollens to the allergic diseases for which they are responsible have indicated that, in most cases, the active principles are associated with the protein fractions. In an examination of the fractions of Timothy grass pollen an attempt was made to isolate a polysaccharide on account of the possibility that such a compound might play a part similar to those of the specific soluble carbohydrates isolated from various pathogenic bacteria.

By use of the methods employed in the isolation of the specific carbohydrate from the tubercle bacillus it has been possible to isolate from the pollen a laevorotatory complex carbohydrate which appears to be of the same chemical type as the corresponding bacterial substances. A preliminary chemical examination has shown that it yields *l*-arabinose, galactose, and a non-reducing acid on hydrolysis with acids, thus displaying a certain similarity to the specific carbohydrate of the tubercle bacillus. The relationship of the polysaccharide to the allergic reactions of the protein fractions of the pollen is being examined.

It is improbable that the polysaccharide is in any way connected with the glucoside dactylin, isolated by Moore and Moore [1931] by direct aqueous extraction of Timothy and Orchard grass pollens, since dactylin gives no pentose reactions. There is very little recorded concerning the occurrence of gum-like carbohydrates in other pollens; Johnson and Rappaport [1932] state that no such substance could be isolated from ragweed pollen, whilst Miyake [1922] describes the isolation of 5·7 % of a pentosan from maize pollen. Black [1931] isolated from the pollen of the giant ragweed a carbohydrate containing 5·2 % N which reacted specifically in the skin and nasal mucosa of ragweed-sensitive persons.

Experimental.

Preparation of the carbohydrate.—10 g. of pollen (*Phleum pratense* collected in 1930) were digested with 4 % sodium hydroxide (200 cc.) on the water-bath for 6 hours. At the end of this period some of the cells were broken, but most were only swollen and slightly ruptured. The liquid was diluted with an equal

volume of water, acidified with acetic acid and filtered. The filtrate was treated with an excess of lead acetate and the precipitate, which contained most of the yellow colouring matter but gave no Molisch reaction, was removed and discarded. Basic lead acetate and sufficient ammonia to basify the liquid strongly were then added and the heavy precipitate, which contained all the substance responsible for the Molisch reaction, was removed, washed with water and suspended in dilute acetic acid. After stirring mechanically for several hours the insoluble material was removed and discarded and the filtrate treated with hydrogen sulphide. The lead-free liquid was then evaporated at 40° to a small volume and poured into ten volumes of ethyl alcohol. The precipitated solid was dissolved in water (8 cc.), treated with an equal volume of methyl alcohol and the precipitated material filtered off. When thus isolated, the material insoluble in 50 % methyl alcohol was only slightly soluble in cold and somewhat more so in hot water. It gave no coloration with iodine and did not reduce Fehling's solution. Upon hydrolysis with acids reducing sugars were formed.

The water-soluble carbohydrate was obtained by pouring the filtrate from the above material into an excess of methyl alcohol (80 cc.) and was further purified by another precipitation from 50 % methyl alcohol.

Two other preparations were made, one from pollen (30 g.) which had been ground in a ball-mill and extracted with dilute saline and the other from pollen (55 g.) which had previously been extracted with acetone. The yields were 1·1 and 1·9 g., respectively. In the last preparation the treatment with sodium hydroxide was prolonged until most of the pollen grains had burst (32 hours).

The preparations formed white powders which were very soluble in water and which changed to gummy masses on addition of a few drops of water. The last two preparations had respectively $[\alpha]_{5461}$ − 42·0° and − 39·1° in 1 % aqueous solution. A fractional precipitation experiment was made to test the homogeneity of the material; a solution of the third preparation (0·2 g.) in water (1·5 cc.) was treated with alcohol (4·5 cc.), the precipitated material (0·06 g.) removed, the filtrate poured into an excess of alcohol (60 cc.), and the second fraction collected (0·13 g.). The first fraction had $[\alpha]_{5461}$ − 34·2° and the second − 39·1°. The fractions contained 0·9 and 0·5 % N. The unfractionated preparation contained 0·2 % P.

The carbohydrate gave strong characteristic reactions for pentoses with hydrochloric acid and phloroglucinol and with hydrochloric acid and orcinol. No reaction for methylpentoses could be obtained by the reaction of Rosenthaler [1909]. No reaction for glycuronic acid was obtained using the modified method of Neuberg and Kobel [1931]. The carbohydrate possessed only a very faint reducing power towards Fehling's solution, but after warming for a few minutes with dilute hydrochloric acid much reducing sugar was formed.

An experiment was made on the rate of hydrolysis of the carbohydrate in sulphuric acid in order to determine whether the sugars were liberated from the complex in stages or not. 7·908 mg. of the carbohydrate were dissolved in 1 % sulphuric acid (200 cc.) and 10 cc. portions of this solution were heated in

a boiling water-bath for periods up to 4½ hours. At the end of the chosen period each tube was cooled quickly and immediately neutralised with the appropriate amount of *N* sodium hydroxide. The amounts of reducing sugars formed were determined as glucose by the Hagedorn-Jensen method.

Tube	Time mins.	% Reducing sugar formed
1	0	4·5
2	5	12·4
3	10	32·6
4	12	34·9
5	20	36·2
6	40	42·8
7	60	45·3
8	80	45·8
9	120	46·5
10	160	48·3
11	220	52·4
12	260	50·1

The values obtained show that reducing sugars are rapidly formed during the first hour of hydrolysis and that after that time a further slow formation of reducing substances occurs which ceases after about four hours.

For the purpose of examination of the products of hydrolysis, 1·1779 g. of the carbohydrate were heated on the water-bath for 2 hours in 100 cc. of 1 % sulphuric acid. The observed rotation ($\lambda = 5461$, $l = 1$ dm.) changed during this time from − 0·46° to + 0·64°. The liquid was then digested with an excess of barium carbonate until neutral to litmus paper. After filtration the liquid, which contained barium ions, was evaporated to a small volume and poured into ten volumes of methyl alcohol. The precipitated material (0·18 g.; Ba, 27·0 % on air-dried material) did not reduce Fehling's solution and gave no naphthoresorcinol reaction. The methyl alcohol-soluble portion was evaporated to a small volume and again treated with methyl alcohol; a trace of insoluble material was removed. The methyl alcohol was finally removed and the residue evaporated several times with water. The residual syrup gave the deep red colour characteristic of pentoses upon distillation with 12 % hydrochloric acid and pouring the distillate into a solution of aniline acetate. Attempts were made to form hydrazones with the following hydrazines: phenylhydrazine, *as*-phenylmethylhydrazine, *as*-benzylphenylhydrazine, *p*-bromophenylhydrazine, and *as*-diphenylhydrazine. Phenylhydrazine gave a small amount of a substance freely soluble in water and crystallising in large needles; it appeared to be quite distinct from mannose phenylhydrazone; *p*-bromophenylhydrazine and phenylmethylhydrazine gave clusters of fine needles similar in appearance to the galactose compounds. Diphenylhydrazine and benzylphenylhydrazine gave comparatively large amounts of crystalline products. More of the diphenylhydrazone was prepared and washed with alcohol and water. It had M.P. 197–199° (decomp.) whilst a sample of authentic *l*-arabinose diphenylhydrazone melted at 199–202° (decomp.); Müther and Tollens [1904] give M.P. 204–205° (decomp.). A solution of this diphenylhydrazone (0·0216 g.) in

pyridine (2 cc.) and alcohol (3 cc.) had $\alpha_{5461} + 0{\cdot}36°$ ($l = 2$ dm.), whilst the authentic specimen (0·0212 g.) had + 0·34° under the same conditions.

Since the appearance of the phenylmethylhydrazone suggested the presence of galactose a portion of the sugar was oxidised with 25 % nitric acid and a small amount of mucic acid was obtained (M.P. 210–212° decomp. when the tube was inserted at 200° and the bath heated fairly rapidly). Oxidation of the original carbohydrate (0·1 g.) also yielded mucic acid (0·0104 g.)

The barium salt (0·1 g.) was subjected to further treatment with 2*N* sulphuric acid (10 cc.) for 16 hours. A small amount of reducing sugar (0·05 g.) was formed and some barium salt recovered (0·03 g.) which gave no naphthoresorcinol reaction. The sugar fraction was divided into two portions one being treated with diphenylhydrazine and the other oxidised with nitric acid. A small amount of mucic acid was obtained, but no arabinose diphenylhydrazone could be isolated.

The recovered barium salt lost 8·17 % of its weight on drying at 110° and contained 30·2 % of barium (calculated on the dried material).

Upon concentration of the alcoholic filtrate from the first precipitation of the carbohydrate a small amount of crystalline material separated in a form which suggested that it might be inositol. After recrystallisation of this substance (0·02 g.) from a small amount of aqueous alcohol it had M.P. 223° alone or mixed with an authentic specimen of inositol.

SUMMARY.

The preparation of a gum-like polysaccharide from the pollen of Timothy grass is described. Upon hydrolysis with acids *l*-arabinose, galactose and a non-reducing acid are produced.

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CLIV. THE PRODUCT OF THE OXIDATION OF GLUCOSE BY GLUCOSE DEHYDROGENASE.

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(*Received July 1st, 1932.*)

Recently, a description was given of the preparation from the livers of various animals of a new enzyme, glucose dehydrogenase, which brought about the oxidation of glucose, apparently with the formation of an acid [Harrison, 1931]. The present paper deals with the isolation and identification of this oxidation product.

Glucose in presence of the dehydrogenase is not oxidised by molecular oxygen, but is oxidised in presence of methylene blue. It was shown that the aerobic oxidation of glucose could be brought about by the dehydrogenase if the oxygen-activating system cytochrome and indophenol oxidase were present. Since it was difficult to prepare sufficient cytochrome for a large scale oxidation of glucose, the oxidation was carried out in presence of air, using methylene blue as an intermediary carrier. The methylene blue is reduced to leuco-methylene blue which is then rapidly oxidised back to methylene blue by the oxygen of the air. The methylene blue thus acts as a carrier between the activated hydrogen of the glucose and the oxygen of the air.

In order to obtain a clue as to the nature of the oxidation product, a number of preliminary experiments were carried out in the Barcroft differential manometer to determine the relationship between the amount of acid formed and the amount of oxygen taken up. The dehydrogenase preparation and methylene blue were put into both vessels of the apparatus, and glucose solution was added to one vessel and an equal volume of water to the other vessel. The enzyme was the dialysed preparation from acetone-liver [Harrison, 1931], equivalent to about 5 g. of acetone-liver in each vessel. The final concentration of methylene blue was 1 in 2500 and of glucose 0·25 *M*. After shaking for about 3 hours at 37°, the oxygen uptake was measured, and the solution in the vessel containing glucose was titrated with $N/50$ NaOH to the same p_H as that of the solution containing no glucose. In spite of the presence of methylene blue, this could be carried out sufficiently accurately by withdrawing a very small amount of solution on the end of a glass rod and using an external indicator such as phenol red. The titration was checked electrometrically using hydrogen electrodes.

As the result of a number of determinations, an average ratio of slightly less than one atom of oxygen to one equivalent of acid formed was obtained. This rendered improbable the formation of a number of acids such as glycuronic,

keto-gluconic or saccharic acid, all of which require more than one atom of oxygen per equivalent of acid. Determination of lactic acid in the solutions with and without glucose had already shown that no lactic acid is formed from glucose during the oxidation [Harrison, 1931]. Experiments on a rather larger scale showed that after removal of protein from the solution, negative nitroprusside and guaiacol tests were obtained for pyruvic acid, and a negative naphthoresorcinol test was obtained for glycuronic acid, though strongly positive tests were given on adding traces of these acids to the solution.

By comparing the amount of precipitate in the solutions with and without added glucose, it was found that the oxidation product of glucose was precipitated by basic lead acetate but not by normal lead acetate. This again excluded as main products of the reaction a number of acids such as malic, tartaric, citric, succinic, malonic and glyceric acids.

On decomposing the basic lead acid precipitate and treating the liberated acid with $CaCO_3$, a calcium salt was obtained which was soluble in water but which was precipitated by alcohol. The acid did not reduce Fehling's solution but reduced hot ammoniacal silver nitrate. In view of the elimination of the acids mentioned above, these properties, coupled with the observation that one equivalent of acid is formed for each atom of oxygen taken up, suggested that gluconic acid might very possibly be the oxidation product. By carrying out the oxidation on a large scale, it was found possible to prepare and isolate derivatives of the acid which were found to be identical with the corresponding derivatives of *d*-gluconic acid.

The methods used for isolation differed somewhat in different experiments, but the technique used in the experiment to be described proved to be the most satisfactory, and details of the other experiments will therefore be omitted.

The glucose dehydrogenase was prepared by water extraction of 450 g. of acetone-liver, followed by dialysis and precipitation by saturation with ammonium sulphate exactly as previously described [Harrison, 1931]. The dry preparation was ground up, put into two large wet collodion sacs and dialysed into solution against distilled water in the ice-chest. After adjusting to p_H 7·4 and centrifuging, the volume of the enzyme solution was 130 cc.[1] 125 cc. of this solution were mixed with 25 cc. of 2*M* glucose (B.D.H. dextrose) and 37·5 cc. of methylene blue (1 in 500), the mixture being put into corked 250 cc. conical flasks and shaken fairly rapidly in a water-bath at 37°. The flasks were opened at frequent intervals and filled with fresh air.

At the same time, two Barcroft differential manometers were used to measure the oxygen uptake. The right-hand vessels each contained 2 cc. enzyme and 0·6 cc. methylene blue. The experimental apparatus contained in addition 0·4 cc. 2*M* glucose, while the control apparatus which measured the residual oxygen uptake of the enzyme preparation received 0·4 cc. water. The side tubes

[1] Recent experiments by Mann [1932] have shown that an activator is concerned in the oxidation of glucose by glucose dehydrogenase. The enzyme preparation described above contains both the dehydrogenase and the activator.

of the vessels contained NaOH to absorb CO_2, and the vessels were shaken in the same water-bath and for the same length of time as the conical flasks.

After four hours, the oxygen uptake in the Barcroft apparatus was measured, and the flasks were opened. Incubation of samples on agar plates showed that the solutions were almost sterile and that the oxidation of glucose was therefore not due to bacteria.

The solutions in the flasks, together with the contents of the Barcroft vessel containing glucose, were brought to approximately p_H 7·6 and the protein was precipitated by the addition of absolute alcohol to a concentration of 80 %. After filtering under suction, the residue was ground up with 100 cc. of water, filtered and washed. The latter filtrate was precipitated with 80 % alcohol and filtered, using a trace of kieselguhr on the filter-paper. The precipitate was ground with a little water, acidified to p_H 4 with acetic acid, filtered, precipitated with 80 % alcohol and again filtered. All the alcoholic filtrates were combined and evaporated down under reduced pressure to about 150 cc.

The solution was brought to p_H 7·6, allowed to stand overnight to permit of the hydrolysis of any lactone, and precipitated by adding a saturated solution of basic lead acetate together with a little NaOH until a drop of either produced no further precipitate (p_H approximately 7·8). The precipitate was suspended in water, stirred well with acetic acid and then brought almost to neutrality by adding NaOH. The precipitate, consisting largely of $PbSO_4$ (from $(NH_4)_2SO_4$ contained in the enzyme preparation) was centrifuged off, washed, and the clear solution and washings were made alkaline and reprecipitated with basic lead acetate. The precipitate was washed twice with water containing a little basic lead acetate. All the washings and the solution left from the first precipitate were combined, freed from lead with H_2SO_4 and $Ba(OH)_2$, concentrated under reduced pressure and reprecipitated with basic lead acetate. (The solutions were never allowed to stand in presence of basic lead acetate in order to avoid the risk of decomposition of any glucose in the solution.) The combined basic lead acetate precipitates were suspended in water and treated with slight excess of H_2SO_4 to remove lead, the excess of H_2SO_4 being exactly removed with baryta. The clear filtrate was evaporated to dryness over solid NaOH in a vacuum desiccator, and most of the acetic acid was removed by reducing the pressure to about 0·5 mm. Hg. (The methylene blue had been almost completely removed at this stage since it was not adsorbed on the lead precipitate.) The residue was dissolved in a little water, stirred with 0·2 g. $CaCO_3$ and left overnight over NaOH. The mixture was then heated on the water-bath for several hours, stirring at intervals, and when nearly neutral a few drops of lime water were added until the solution was permanently just neutral to phenol red. The solution was filtered and the calcium salt was precipitated with 80 % alcohol in a total volume of about 50 cc. After standing overnight in the ice-chest, the precipitate was centrifuged off and dissolved in about 2 cc. of warm water, and warm alcohol was added to a concentration of 30 %. On standing overnight the calcium salt crystallised out and was filtered and washed with a little

cold 30 % alcohol and dried over H_2SO_4. The mother-liquor and washings were evaporated down and again allowed to crystallise. By repeating this process four times, assisting crystallisation each time by seeding the solution with a crystal from the previous crop, a total of 255 mg. of crystalline salt was obtained, the crystals consisting of clusters of microscopic needles resembling the highly characteristic crystals of the calcium salt of gluconic acid.

By heating a portion of the free acid on the water-bath with phenylhydrazine and acetic acid [Fischer and Passmore, 1889], crystals were obtained on cooling which, after dissolving in hot water, decolorising with a little charcoal and recrystallising once from very dilute acetic acid and three times from hot water, commenced to decompose at 196° on rapidly heating and melted completely at 200°. A sample of the phenylhydrazide of gluconic acid prepared from an authentic specimen of the acid melted at the same temperature, and the melting-point was not changed by mixing the two specimens together. The two samples of crystals were apparently identical under the microscope, being microscopic, square-ended prisms with straight extinction. Further confirmation that the acid is gluconic acid was obtained by preparing the cinchonine salt which after recrystallising from 95 % alcohol had M.P. 185–186°. Micro-analysis (Schoeller) of the phenylhydrazide : C, 50·38, 50·50; H, 6·22, 6·28 %. (Calculated for $C_{12}H_{18}O_6N_2$: C, 50·35; H, 6·29 %.)

There appears to be no doubt, therefore, that gluconic acid is formed by the oxidation of glucose in presence of glucose dehydrogenase.

In order to determine whether the acid were *laevo-* or *dextro*-gluconic acid, 150 mg. of the dry, recrystallised Ca salt were dissolved in water, made up to 5·0 cc., and the rotation measured in a 1 dm. tube at 20°. Taking the average of a number of closely agreeing readings, the specific rotation $[\alpha]_D^{20^\circ}$ was calculated to be + 8°. The values given in the literature for Ca *d*-gluconate range from + 6° to + 10°. As would be expected, therefore, the *dextro*-form of gluconic acid is formed by the oxidation of *d*-glucose by the enzyme.

The oxygen uptake during the oxidation was equivalent to the formation of 424 mg. of calcium gluconate (after subtracting the residual oxygen uptake of the enzyme alone). The amount actually isolated in the crystalline state was 255 mg. which is equivalent to just over 60 % of the oxygen uptake observed. In dealing with such small amounts, the isolation is by no means quantitative, and it seems quite likely, therefore, that *d*-gluconic acid is the sole product of this oxidation of glucose.

Discussion.

The oxidation of glucose at the potential aldehyde group with the formation of gluconic acid is readily brought about by a number of oxidising agents and would seem at first sight a probable method of breakdown of glucose in the body. The results reported in the literature of feeding and perfusion experiments with gluconic acid are rather contradictory, but there appears to be a certain amount of evidence that some utilisation of gluconic acid takes place. Experi-

ments in which unchanged gluconic acid was recovered in the urine after feeding the acid may possibly be explained by the rapid elimination of the acid by the kidneys after feeding in relatively large doses, this preventing a quantitative utilisation of the acid.

While, therefore, gluconic acid may be a normal intermediary in the oxidation of glucose, an alternative possibility might be that the gluconic acid which has been isolated in these experiments may arise from some labile oxidation product of glucose which normally, in the intact cell, becomes further oxidised instead of appearing as gluconic acid. A similar hypothesis has been brought forward by Meyerhof [1930] to explain the conversion by muscle enzyme of glycogen to lactic acid *via* hexosemonophosphate. In this case, although hexosemonophosphate can be isolated during the reaction, yet glycogen is broken down by the enzyme more rapidly than the supposed intermediary, hexosemonophosphate. Meyerhof explains this by assuming that the true breakdown path is *via* an unknown labile form of hexosemonophosphate which, if not immediately converted into lactic acid, may appear as stable hexosemonophosphate.

I put these possibilities forward only as mere suggestions, but in view of the experiments I have described and of the high concentration of glucose dehydrogenase in the liver, it seems difficult to escape from the conclusion that in the body, part, at least, of the glucose metabolised must go through gluconic acid or some precursor of this acid during its oxidation.

It is interesting that glucose dehydrogenase and glucose oxidase, the enzyme prepared from *Aspergillus niger* by Müller [1928], both convert glucose into gluconic acid; for, as previously pointed out [Harrison, 1931], the two enzymes differ fundamentally in properties, the oxidase being able to utilise oxygen but not methylene blue as the hydrogen acceptor, while the dehydrogenase can use methylene blue but not oxygen directly.

SUMMARY.

The product of the oxidation of glucose by glucose dehydrogenase has been isolated and shown to be *d*-gluconic acid.

The amount of acid isolated as the crystalline calcium salt was equivalent to over 60 % of the amount formed as calculated from the oxygen uptake, and *d*-gluconic acid is probably the sole product of the oxidation.

The author wishes to express his thanks to Prof. E. Mellanby for his interest in this work.

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CLV. CASTRATED RATS FOR THE ASSAY OF TESTICULAR HORMONE.

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In a previous paper[1] [Korenchevsky, 1932] the increase in weight of the prostate with seminal vesicles of injected castrated rats by at least 40 % as compared with those of control uninjected rats was proposed as a quick and sensitive method of assay of testicular hormone.

Since, after castration, the weights of the penis and thyroid also decrease, whilst the hypophysis, adrenals and body fat increase in weight, the suggestion was made that, for a test of the complete activity of the testicular hormone, it was necessary to obtain a return towards normal of these organs.

The importance for accurate assay of using litter-mate castrated rats was stressed. In this case the litters were to be divided into three groups, (1) controls uninjected, (2) rats injected with a standard preparation, or a previously prepared preparation, (3) rats injected with the new preparation of hormone. The chief difficulty in the assay is that, up to the present time, such a standard preparation is not available. Even if it were available it would with difficulty be kept at a constant strength owing to the instability of the testicular hormone, due chiefly to oxidation. In any case it would be necessary to test the standard preparation from time to time.

The question, therefore, arises under what conditions the castrated rats of different litters will give the nearest comparable results in assay.

In the previous paper was noted the importance of using rats in litters from the same stock, reared on the same diet, of the same age, at the same date after castration, and kept during the experiment on the same complete synthetic diet.

The present communication contains a study of the post-castration changes of the organs mentioned above in different litters of male rats, in relation to sexual maturity at the time of castration of the rats and to the number of days elapsing between castration and the examination of the rats.

[1] On p. 420 of this paper, the weights of adrenals, thyroids and hypophysis must be divided by 10, the decimal points having been inadvertently omitted.

PRESENT INVESTIGATION.

Eighty-eight castrated rats belonging to 48 litters (40 litters in pairs and 8 litters singly) have been arranged in Tables I and II according to sexual maturity at the time of castration and the interval in days between castration and the time at which the rats were killed. These rats were used as the controls of several other experiments. All the rats belonged to the Lister Institute stock and were reared on the same diet.

The 50th day after birth was chosen as the age of sexual maturity. In the Lister Institute, sexual maturity, as judged by fertility, has been exceptionally noted as early as the 38th day after birth, and frequently, but not generally, at the 50th to 56th day (unpublished results of Miss H. H. Smith). The 50th day was, therefore, taken as the earliest frequent date of sexual maturity. This is in agreement with most observations on different stocks. Allen [1918], using rats from the Wistar Institute, found the first ripe spermatozoa in testes between the 36th and 40th day; Andersen [1932] gives the age of sexual maturity as about 60 days as judged by the finding of motile spermatozoa in the epididymis. The rats, however, about 50 days old sometimes had large numbers of non-motile spermatozoa. Hewer [1916] alone gives a later date for sexual maturity in his stock, namely, at about 70 days old.

It is clear from Table I that each of the four groups of rats castrated in an immature state, arranged in relation to the number of days after castration, represents a particular age with comparatively small deviations from the

Table I. *The number and age of the castrated male rats in the different groups and their average ages (with the deviations from the average in brackets).*

	No. of days after castration when killed							
	20–30		31–70		71–90		136–182	
	No. of Rats	Age in Days	No. of Rats	Age in Days	No. of Rats	Age in Days	No. of Rats	Age in Days
Castrated when sexually immature	8	58 (+11) (−4)	13	95 (+18) (−15)	6	111 (+12) (−10)	11	203 (+13) (−28)
Castrated when mature	22	88 (+12) (−11)	19	137 (+64) (−36)	8	184 (+12) (−16)	1	286

average age for each group. In the rats castrated after maturity these deviations are greater in the second group. As would be expected, the longer after castration the rats were killed, the older they were.

The weights of the organs were calculated per 200 g. of body weight for each rat. When the litter was represented by a pair of rats the average weight of each organ was found for this litter. From these weights a general average weight of the organs for each group of litters was made and tabulated, together with the maximum deviations from the general average.

Thus the greatest fluctuations in each group between the different litters are shown for each organ. In addition the variations between the different

groups in relation to sexual maturity at castration and the number of days elapsing between castration and the killing of the rats are shown.

In relation to the thymus and penis the following facts must be taken into consideration. The thymus was not dissected in the earlier experiments and, therefore, the averages are obtained for only 28 rats belonging to 14 litters, that is from a small number of observations.

With regard to the penis the technique of dissection of this organ was changed, and in some of the first litters, which unfortunately were not noted, the penis was dissected together with the enlargement at the root by which it is attached to the pelvic bones. Later it was found that the weighing of the penis is much more accurate if the section was made just above these enlargements[1]. The weights by the second method are, therefore, smaller than by the former method. Hence the most reliable data with regard to the sexual organs are those for prostate with seminal vesicles. It is also necessary to note that the fourth group of "mature" litters was represented by one litter only and, therefore, no conclusions can be drawn for this group.

Sexual organs.

From Table II it is clear that the period at which castration is performed, *i.e.* before or after sexual maturity, is of great importance with regard to the

Table II. *The average weights of the organs and retroperitoneal fat per 200 g. of body weight of castrated male rats belonging to different litters in relation to the sexual maturity at the time of castration and the number of days after castration at death (with the maximum percentage deviation from the average in brackets).*

Organs	Condition of rats at castration	Days elapsed between castration and killing			
		20–29	30–67	70–89	136–182
Prostate and seminal vesicles (mg.)	Immature	103 (10·7)	60 (26·7)	55 (18·2)	60 (23·3)
	Mature	154 (24·0)	152 (38·1)	180 (16·1)	199 —
Penis (mg.)	Immature	85 (20·0)	90 (52·2)	59 (27·1)	65 (83·1)
	Mature	110 (36·3)	183 (59·5)	221 (10·0)	224 —
Thymus (mg.)	Immature	635 (12·8)	495 (6·3)	390 —	— —
	Mature	447 (29·3)	256 —	— —	— —
Adrenals (mg.)	Immature	66·6 (10·7)	47·4 (27·2)	41·1 (12·9)	42·4 (20·7)
	Mature	46·1 (21·0)	40·0 (41·2)	38·3 (6·5)	43·9 —
Thyroids (mg.)	Immature	22·0 (20·4)	19·8 (42·9)	19·2 (18·2)	17·9 (38·5)
	Mature	20·5 (20·0)	17·8 (47·4)	19·6 (6·6)	15·4 —
Hypophysis (mg.)	Immature	11·8 (11·9)	9·0 (25·5)	8·9 (5·6)	8·6 (11·6)
	Mature	8·7 (25·3)	8·6 (66·3)	8·2 (26·8)	7·1 —
Retroperitoneal fat (g.)	Immature	2·79 (19·3)	6·65 (47·3)	8·00 (19·6)	10·88 (25·9)
	Mature	6·87 (47·0)	7·22 (58·3)	7·34 (29·2)	4·89 —

degree of atrophic changes in the sexual organs. If the rats are spayed before maturity the atrophic changes are greater than if the operation is performed after maturity. At the same time the weight of the prostate with seminal

[1] It has now been found that weighing the dissected penis is more accurate after previous fixation in Bouin's fixative than without fixation (*i.e.* the penis should be treated in the same way as the other organs).

vesicles per 200 g. of body weight remains constant from the end of the first month after castration, being practically the same at six months after castration. During the first month after castration the weight is a little greater.

The fluctuations between the different litters of the same group are not very large, the maximum deviation not exceeding 27 %, and the average deviations being 8·5, 15·0, 10·9 and 15·0 % for the four groups respectively.

If the rats are castrated after sexual maturity, the fluctuations are similar, being again greatest in the second group (31–70 days after castration); the largest deviation was about 38 %, the average deviations being 9·4, 19·3 and 9·4 % for the three first groups respectively.

The somewhat greater fluctuations in the weight of the penis may be due to the use of two methods of dissecting the penis, as mentioned above.

Forty-eight litters yield too small a number of observations from which to select adequate material for the study of the influence of age at castration within the two large divisions grouped as before and after sexual maturity. However, eight litters can be chosen to indicate this latter influence since all were killed at about the same time after castration (from 64 to 69 days after the operation).

1. *Before sexual maturity.* The average weights of the prostates with seminal vesicles of three litters castrated 22–25 days after birth were 46, 50 and 60 mg. per 200 g. of body weight respectively, whilst the average weights of these organs were 76 mg. in each of two litters castrated at 49 days after birth, *i.e.* at a date close to maturity.

2. *After sexual maturity.* These organs in two litters castrated at 64 and 74 days after birth weighed 112 and 126 mg. per 200 g. of body weight, whilst in the litter castrated at 134 days old the weight was 210 mg.

Taking into consideration the fact that maturity may vary from the 36th to the 70th day, as mentioned above, and that the 50th day was taken only as a frequent early date of sexual maturity, some of the rats within this period might actually be operated on before, others during and others after, maturity. Therefore, on the average within this period of age the changes observed may be nearly similar.

The thymus in rats, as in other mammals, reaches its greatest weight at sexual maturity and from then the normal process of involution begins. Castration prolongs the period of growth of the thymus and inhibits involution. In our rats the weight of the thymus per 200 g. of body weight was greater in those castrated before, than in those castrated after, sexual maturity (Table II), which is in agreement with the fact mentioned above.

Retroperitoneal fat increased in amount in the "immature" group with increase in the number of days elapsing between castration and the date of killing of the rats. This difference was not so distinctly marked in the "mature" group.

In the rats killed 20–30 days after castration the weight of fat was greater in the "mature" group (6·87 g.) than in the "immature" group (2·79 g.), which may be due to the greater average age of the rats in the former group.

Adrenals, thyroids and hypophysis. The largest weights per 200 g. of body weight of the adrenals, and to a less degree of the thyroids and hypophysis, of castrated rats were observed in the "immature" group about 20–30 days after castration, gradually decreasing with the increase in number of days after castration. This difference was less marked in "mature" litters. The last group of the latter contained one litter only and the figures must, therefore, be taken with reservation.

The average figures obtained are in good agreement with those previously published [Korenchevsky, 1930, Tables 1–8].

Discussion.

Taking into consideration all the data relating to the changes in weights of organs calculated for the same unit of body weight the following facts are noticeable.

The greatest differences were observed (*a*) between the litters castrated before sexual maturity and those castrated after, and (*b*) between the litters killed during the first month after castration and those killed $4\frac{1}{2}$ months and later after castration.

About $2\frac{1}{2}$ months after operation the changes obtained became more or less stationary (prostate, penis, adrenals) or continued to develop more slowly (fat, thyroids, hypophysis) so that the similarity was found to be greatest in the litters killed $2\frac{1}{2}$–6 months after castration.

The variations in nearly all the organs were greatest in the litters killed 31–70 days after castration, probably owing to uneven rates of development of the changes towards the final state during this period.

Thus the changes produced by castration may be expected to be most nearly similar in different litters if the rats are operated on at the same number of days after birth.

Since the time of operation, before or after sexual maturity, influences profoundly the changes obtained, and since the exact date of maturity in different litters and stocks varies from the 36th to the 70th day, it is advisable to castrate the rats before the 30th day if immature age is chosen for operation, or after the 70th day if sexually mature rats are wanted for operation.

It is also necessary to choose a definite period after castration for beginning the experiment.

Taking into consideration all the limitations mentioned above it must be admitted that in rats belonging to different litters the variations in the weights of the organs investigated were not great provided the rats belonged to the same group.

The inter-relationship between sexual maturity and castration is probably due to the fact that castration before maturity (i) hinders the development and growth of sexual and some other organs, and (ii) produces changes in the already developed organs (*e.g.* atrophy of the sexual organs). After maturity only this latter effect of castration operates.

SUMMARY.

1. Calculated per the same unit of body weight the weights of retroperitoneal fat, sexual and endocrine organs of 88 castrated male rats belonging to 48 different litters were compared.

2. On the average the variations observed between different litters were not great provided the litters belonged to the same group in relation to sexual maturity at the date of castration (before or after sexual maturity) and in relation to the time elapsing after castration.

3. Because of the uncertainty of the exact age of sexual maturity in rats belonging to different litters and stocks, it is advisable to operate on rats before the 30th day after birth if the operations are to be made on immature rats, and after the 70th day if sexually mature animals are to be castrated.

4. In order to obtain accurate and comparable results all the other precautions described in the previous paper must be taken.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled me to carry out this work and to them my thanks are due.

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CLVI. THE RESPONSE OF CASTRATED MALE RATS TO THE INJECTION OF TESTICULAR HORMONE.

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A method of testing the activity and strength of testicular hormone on castrated rats of the same litter was previously described and conditions were suggested for applying the same method of assay of testicular hormone to rats belonging to different litters [Korenchevsky, 1932, 1]. For this latter purpose it is most important to ascertain (1) how castrated rats belonging to different litters will respond to the same dose of testicular hormone, (2) what conditions must be observed to obtain comparable and accurate results.

In a previous paper [Korenchevsky, 1932, 2] it was shown that the weights of the sexual and endocrine organs and of retroperitoneal fat, when calculated per 200 g. of body weight, did not vary greatly in different castrated litters, bred and kept in the same conditions provided they were operated on at the same period in relation to sexual maturity, *i.e.* before or after puberty, and also that the litters were killed at the same period after castration.

In this paper a study is made of the influence of these two factors on the changes in rats injected with testicular preparations, in order to ascertain under what conditions comparable results for the assay of the hormone when using different litters may be obtained.

The response of the rats of the same litter to different doses was also compared in a few experiments so as to obtain an indication of the sensitivity of the test to differences in the dose.

Technique.

Experiments were performed on 67 rats belonging to 17 litters, all of which came from the stock of the Lister Institute and were reared and kept on the same diet. During the experiments a special paste was given, and all the other details of technique for the assay of testicular hormone were the same as those described elsewhere [Korenchevsky, 1932, 1]. The amount of paste consumed daily was recorded. Castration was performed at different ages in different litters. All the data mentioned above, together with the changes in body weight of the rats, are summarised in Table I.

Table I. *Age and average changes in appetite and body weight of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Age in days: At castration	Age in days: At beginning of injections	No. of days after castration injections started	Change in wt. per 200 g. of initial wt. of rat: Control rats g.	Change in wt. per 200 g. of initial wt. of rat: Injected rats g.	Paste intake per day per 200 g. of body wt.: Control rats g.	Paste intake per day per 200 g. of body wt.: Injected rats g.
1	4	72	82	10	+50	+75	27	28
2	3	70	80	10	+35	+38	20	17
3	4	57	67	10	+92	+84	29	26
4	4	57	67	10	+74	+61	27	25
Average	4	64	74	10	+63	+64	26	24
5	3	25	81	56	+ 7	+14	20	22
6	4	23	79	56	+20	+27	22	20
Average	4	24	80	56	+14	+20	21	21
7	4	63	82	19	+ 4	+25	18	21
8	5	71	90	19	+16	+26	20	21
9	4	57	76	19	+28	+15	25	19
Average	4	64	83	19	+16	+22	21	20
10	5	74	103	56	+ 2	+ 5	16	14
11	3	23	85	62	+44	+16	23	19
12	3	67	93	26	+51	+14	23	16
13	3	44	116	72	+ 7	+ 1	16	11
14	2	150	279	129	− 2	− 7	14	10
15	2	39	168	128	+ 3	− 1	13	14
16	3	76	148	72	+ 4	− 1	15	11

The testicular extracts Nos. 126 and 130 were prepared by the "dry" method previously described [Korenchevsky, Schalit and Graetz, 1932] and dissolved in sesame oil. The extracts Nos. 4, 89 and 92 were prepared by Koch and Gallagher's method [1929] as far as the stage of extraction from hexane solution with 70 % alcohol. Extract 111, prepared by the same method, was purified to the last stage. These last four extracts were emulsified in 1 % gum acacia. All the extracts were sterilised for 30 minutes on three consecutive days in the steamer at 100° and kept in sealed ampoules in the cold store at 0°. No induration or inflammation was in any case produced by the injections but when the extract was dissolved in sesame oil some amount of unabsorbed oil was always found at autopsy in the subcutaneous tissue at the sites of more recent injections.

The influence of the injections in different litters.

These results are summarised in Table II. The experiments on litters 1 to 10 were designed to show this influence. Those on litters 11 to 16 were preliminary experiments for the purpose of testing the strength of a testicular preparation. In these experiments the difference in the response of different litters became evident. In addition to the organs given in Table II the other endocrine organs and the retroperitoneal fat were weighed. These are not included in the Table, since the amount of the testicular preparation injected

Table II. *The influence of the injection of testicular hormone on the weights of the sexual organs and thymus, calculated per 200 g. of body weight, of the rats in each group of each litter.*

Extract injected and length of period of injections	No. of litter	Prostate and seminal vesicles (mg.)			Penis (mg.)			Thymus (mg.)		
		Control rats	Injected rats	Increase %	Control rats	Injected rats	Increase %	Control rats	Injected rats	Decrease %
No. 126	1	137	206	50·4	85	110	29·4	382	341	10·7
20 mg.	2	160	280	75·0	88	114	29·6	339	328	3·2
10 days	3	150	253	68·7	100	118	18·0	471	384	18·4
	4	153	274	79·1	97	124	27·8	467	441	5·6
	Average	150	253	68·3	93	117	26·2	415	374	9·5
No. 130	5	60	145	141·6	43	99	130·2	525	415	21·0
12 mg.	6	50	129	158·0	46	95	106·5	464	335	27·8
10 days										
	Average	55	137	149·8	45	97	118·4	495	375	24·4
No. 130	7	157	298	89·8	126	136	7·9	516	325	37·0
12 mg.	8	157	300	91·1	105	129	22·9	359	277	22·8
10 days	9	121	267	120·6	87	108	24·1	460	323	29·8
	Average	145	288	100·5	106	124	18·3	445	308	29·9
No. 130	10	126	221	75·4	104	123	18·3	256	185	27·7
12 mg.										
10 days										
No. 4	11	46	114	147·8	—	—	—	—	—	—
20 mg.	12	184	260	41·3	—	—	—	—	—	—
10 days										
No. 89	13	65	146	124·6	—	—	—	—	—	—
20 mg.	14	199	291	46·2	—	—	—	—	—	—
7 days										
No. 92	15	50	100	100·0	—	—	—	—	—	—
20 mg.	16	110	171	55·5	—	—	—	—	—	—
6 days										

and the length of the period of injections were not sufficient to produce marked changes. During these experiments dissection and investigation of the changes in the thymus were begun. It was found that, like the sexual organs, the thymus is also sensitive to the small amounts of testicular preparations used for the test.

Experiments with testicular extract 126.

The same amount of extract 126 (20 mg. per day per rat) was injected daily for 10 days into two rats of each of the litters Nos. 1 to 4. The remaining litter-mates served as the controls. As can be seen from Table I, the rats of all the litters were castrated at the age of sexual maturity; the injections were made for 10 days and the rats killed 20 days after castration.

The weight of the prostate with seminal vesicles increased in the injected rats, as compared with the controls, on the average by 68·3 %, the deviations from this average not exceeding 26·2 %. The maximum difference in the increases was between the litters 1 and 4, being about 57 % greater in the latter litter than in the former.

The response of the penis to the injections was smaller than that of the

prostate and seminal vesicles, the increase being on the average 26·2 %, with a maximum deviation from the average of 31·3 %.

The response of the thymus was still less, its reduction averaging 9·5 %, with a maximum deviation of 93·6 %.

Experiments with extract 130.

Three groups of litters were injected with extract 130, using 12 mg. per rat daily for 10 days. Litters 5 and 6 belonged to the first group of rats castrated before sexual maturity at the age of 23 and 25 days. Litters 7, 8 and 9 formed the second group of rats castrated after maturity and injected 19 days after castration. The third group, containing only litter 10, was also operated on after maturity, but the injections were made much later, namely, 56 days after castration.

The increase in the prostate and seminal vesicles on injection was much greater in the first group (litters 5 and 6) than in the second and third groups (litters 7–10), as was also the case with regard to the increase in weight of the penis. The decrease in the thymus was on the average nearly the same in all three groups.

This experiment indicates quite clearly that, with regard to the increase in weight of the sexual organs on injection with the hormone, the reaction is much greater in rats castrated before, than in rats castrated after, sexual maturity.

The results are influenced more by the date of operation in relation to maturity than by the length of time between the operation and the beginning of the injections.

Thus the increases in weight of the prostate with seminal vesicles in litters 7 and 8 are very close to that of litter 10, being 89·8 %, 91·11 % and 75·4 % respectively. The maximum difference between litters 8 and 10 was about 21 %, whilst litter 9, castrated at 57 days, *i.e.* close to the date of maturity (perhaps not even having reached maturity), showed a greater deviation from litters 7 and 8, to which group litter 9 belongs, the weight of prostate with seminal vesicles being 34·3 % greater in litter 9 than in litter 7. The maximum deviation from the average, however, of the prostate with seminal vesicles in the second group was 20 %.

Experiments with extracts Nos. 4, 89 and 92.

Six litters were used for these experiments Nos. 11 to 16. Each of the extracts was injected into two litters, one castrated before and the other after sexual maturity (Table II). Although in some litters there were only two rats, one injected and the other serving as the control, the results are identical with those of litters 5–10, *i.e.* in the rats castrated before maturity the increase in the prostate with seminal vesicles on injection was much greater than in those castrated after maturity.

The injection of different doses into rats of the same litter.

These experiments were performed on three litters, Nos. 5, 7 and 17, and the results are summarised in Table III. The results of the injection of litters

Table III. *Percentage changes in the weights of organs after the injection of different doses of testicular extract into groups of rats belonging to the same litter, the food intake per day per 200 g. of body weight and the change in body weight per 200 g. of initial weight.*

		Prostate and seminal vesicles			Penis			Thymus		
			Rats given large dose			Rats given large dose			Rats given large dose	
No. of litter	No. of rats in litter	Rats given small dose	Observed increase	Expected increase	Rats given small dose	Observed increase	Expected increase	Rats given small dose	Observed decrease	Expected decrease
5	6	+142	+263	+213	+130	+191	+195	-21	-27	-31
7	6	+ 90	+129	+135	+ 8	+ 8	+ 12	-37	-43	-56
17	6	+ 39	+ 76	+ 77	+ 1	+ 19	+ 2	—	—	—

		Paste intake per day per 200 g. body weight			Gain in body weight per 200 g. of initial weight			
No. of litter	No. of rats in litter	Control rats	Rats given small dose	Rats given large dose	Control rats	Rats given small dose	Rats given large dose	Extract and daily doses injected
5	6	21	22	21	+ 7	+14	+25	No. 130
								12 mg. 18 mg.
7	6	18	21	22	+ 4	+25	+33	No. 130
								12 mg. 18 mg.
17	6	21	18	18	+31	+16	+17	No. 111
								4 mg. 8 mg.

5 and 7 with the small dose will also be found in Table II), since this small dose and the test preparation, No. 130, were the same as were used for litters 5 to 10. The larger dose of this extract injected was 18 mg. per day.

Litter 17 was castrated at 65 days old, and injections were started at 81 days old, or 16 days after castration, the period of injection being 12 days. Extract 111 was injected into 2 rats of this litter in the daily dose of 8 mg. per rat, the two remaining rats serving as uninjected controls.

In Table III the first two columns for each organ give the changes % actually observed after the injection of two different doses; an increase in the case of prostate with seminal vesicles, a decrease in the case of thymus. In the third column is given the expected increase or decrease with the larger dose, assuming that the increase in the change would be in direct proportion to the increase in the dose. Thus, in litter 17 (see Table IV) the average weight of the prostate with seminal vesicles of the control rats was 191 mg. After the injection of 4 mg. per day per rat the average weight of the prostate with seminal vesicles of the two injected rats was 265 mg., an increase of 74 mg.

Therefore the double dose would be expected to produce an increase of 148 mg. though so close an agreement with the actual result of 146 mg. was probably fortuitous.

In litter 7, however, the expected and actual increases are again very close; though in litter 5 they are more divergent, the actual increase being 263 % while the expected increase was only 213 %.

It is necessary to add that this close agreement is only true for the comparison of the averages of each group. Variations are always present between the individual rats of the group, as was shown in a previous paper [Korenchevsky, 1932, 1].

For instance, in these two litters 7 and 17, giving the striking results mentioned above, the weights of the prostate with seminal vesicles, when calculated per 200 g. of body weight, in the individual rats of the three groups, were as follows:

Table IV.

Weights in mg.

		Litter 7	Litter 17
Two rats of control group		165	193
		149	189
	Average	157	191
Two rats injected with small dose of hormone		314	280
		281	250
	Average	298	265
Two rats injected with large dose of hormone		409	298
		308	376
	Average	359	337

This shows the importance of using at least two rats for a group, in which case the reliability of the average obtained from the data given even by only two rats, as has already been shown in a previous paper [Korenchevsky, 1932, 1], is usually satisfactory, provided that more than one litter is used for the conclusions.

Discussion.

Although the number of experiments performed was not large, very similar results were obtained in all cases. At the same time the corroboration of the results by a large number of experiments on the stocks of rats of different laboratories is necessary.

Some facts, however, became evident. Thus, in castrated rats the prostate and seminal vesicles, weighed together, are the most sensitive organs for the assay of testicular hormone.

The response of these organs is much greater, *i.e.* they are much more sensitive, if the rats are castrated before than after sexual maturity. It seems advisable, therefore, to use rats castrated before sexual maturity for the assay.

Since the exact date of maturity is uncertain it is necessary to castrate the rats before the 30th day after birth and to use only such castrated rats for the tests.

The data are insufficient to enable us to decide what time should elapse after castration before the rats are used for assay.

As has been stated before, however [Korenchevsky, 1932, 2], from the beginning of the second month to 6 months after castration, when this was performed before maturity, the weights of the atrophied prostate and seminal vesicles, calculated for the same unit of body weight, were found to be practically constant. For the other organs, however, more nearly constant results are not obtained until 2½ months after castration. Should it be found that rats, within this period after castration, respond to the same degree to the injections, this period would perhaps be most suitable. In those experiments in which injections of different doses of testicular hormone were made, on the average a remarkably exact correlation was noted, in the form of a direct proportion between the increase of the dose and in the increase in the weight of the atrophied prostate with seminal vesicles. If this is found to be corroborated by more numerous experiments, it will be possible to calculate sufficiently nearly, from any dose injected in a preliminary experiment, the dose necessary for the assay injections. In this case the standardisation of the hormone will be easier than by the comb-growth method, in which only small doses give reliable comparable results.

It seems at the present moment, on the basis of the experiments described in this and the papers mentioned above, that a method of assay on rats belonging to different litters and a rat unit of testicular hormone can be suggested. The following definition is proposed only as a foundation for testing the method experimentally for simplicity, reliability and degree of exactness and for comparison with other methods.

As a rat unit, we suggest the minimum daily dose of testicular hormone injected during 7 consecutive days into at least 3 litters of rats castrated before the 30th day after birth, which will produce, on the average, an increase of 40 % in the weight of the prostate with seminal vesicles as compared with the weight of these organs in the uninjected litter mates.

For the assay the emulsification (or solution if possible) of testicular hormone in watery solvents will be preferable, since when the testicular hormone is injected dissolved in oil uncertain amounts of unabsorbed oil were always found in the subcutaneous tissue at the places of more recent injections. This probably indicates that some of the hormone injected during the assay was not absorbed. The amount of this unabsorbed hormone in oil may vary and become a source of error. In our experiments an attempt to decrease this error was made by increasing the period of injections to 10 days, thus allowing time for the complete absorption of the extract injected during approximately the first 7 days, *i.e.* the greater part of the injected material.

It is clear from Table II that although the increase in weight of the penis and decrease in weight of the thymus confirm the activity of the extract, and therefore, it is advisable to weigh them also, the response is not sensitive and the variations observed are sometimes large as compared with those of the

prostate with seminal vesicles. They are not, therefore, suitable for a quantitative assay.

In Tables I and III the figures showing the increase in weight and the appetite of the injected as compared with the uninjected rats are given. Marked simultaneous decreases of these in the injected rats usually indicate the presence of depressing substances in the preparation. This was noted for instance in litters 11 to 17, which were injected with the first extracts we prepared.

Loss in weight without a diminished, or even with an improved, appetite may indicate an increased metabolism.

In litters 1, 5, 6, 7 and 8 (Tables I and III) better growth was obtained after the testicular injections, especially when larger doses were injected (Table III).

A large reduction in the appetite of the injected rats also checks the normal rate of increase of the weight of prostate and seminal vesicles.

Summary.

1. Using 67 rats belonging to 17 litters a study has been made of the response of rats belonging to different litters to the same dose of testicular extract, and also of the response of rats to different doses of the same testicular preparation.

2. The conditions necessary for the assay of testicular hormone suggested in previous papers were corroborated.

3. In castrated rats the most sensitive organs suitable for the assay of testicular hormone are the prostate with seminal vesicles.

4. Although the penis and thymus respond to the injections simultaneously with the prostate and seminal vesicles, the response is less in degree, is more variable and is less sensitive.

5. The increase on injection in the weight of the atrophied prostate with seminal vesicles is much greater in rats castrated before, than in rats castrated after, sexual maturity.

6. In rats belonging to different litters, injected at a similar period after castration with the same dose of the same testicular extract, the variations in the increase in weight of the prostate with seminal vesicles were not large provided the litters were operated on at the same time in relation to the date of sexual maturity, *i.e.* before or after maturity.

7. In three experiments a very close correlation of direct proportion between the increase of the dose of the injected hormone and the degree of the average increase of prostate with seminal vesicles was obtained.

8. On the basis of the results mentioned in this and other papers a suggestion has been made of a possible method of assay of testicular hormone preparations on rats belonging to different litters.

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CLVII. THE ABSORPTION OF NITROGEN AND OF FAT FROM THE ALIMENTARY CANAL OF RATS KEPT ON A VITAMIN A-DEFICIENT DIET.

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The factors responsible for the pathological condition leading to failure of normal growth in rats kept on a vitamin A-deficient diet are not exactly known. Since the appetite of such rats is less than that of normal rats, the most obvious explanation of the decreased rate of growth would be an insufficient food intake. A second possible cause of the disturbance of rate of growth might be a decreased absorption of food from the alimentary canal. This latter is suggested by the fact that frequently gastro-intestinal disturbances are associated with this dietetic defect. In this paper a preliminary investigation of these two suggestions is described.

Technique.

A litter of four male rats was used for the experiment. Two of the rats were placed on a vitamin A-deficient paste diet and two on the same paste supplemented by cod-liver oil, given by pipette. In addition to the paste, the vitamin A-deficient rats were given as many milligrams of cotton-seed oil as the control rats received of cod-liver oil.

The rats were placed on the experimental diet at the age of 33 days and kept in the usual cages for 24 days, by which time the rate of growth of the rats on the deficient diet had begun to decrease. At the age of 58 days, on the 25th day of feeding with the experimental diet, the rats were placed in metabolism cages in a special room kept at a constant temperature, and all the necessary precautions for accurate metabolism estimations were taken, as described in a previous paper [Korenchevsky and Dennison, 1932]. The paired feeding method was used in these experiments. The rats on the vitamin A-deficient diet, Nos. 1756 and 1757, however, were allowed to consume the paste *ad libitum* since the main object of the experiment was an estimation of the appetite and the absorption of the food. The control rats, Nos. 1758 and 1759, received the same quantity of food, the amount given being equal to the amount eaten on the previous day by rats 1756 and 1757 respectively.

Although the daily food intake was not kept constant some conclusions on nitrogen metabolism can be made owing to the fact that the rats were paired and that the food intake per 200 g. of body weight of the controls was little less than that of the rats on the vitamin A-deficient diet.

In order to avoid decreasing the amount of food given to the control rats on complete diet to such an extent as to be injurious to their condition, each pair was killed on the day that the vitamin A-deficient rat of the pair showed considerable loss of appetite. As can be seen from the composition of the paste, given below, the content of vitamin B in the form of dried yeast was very high. On such a vitamin A-deficient diet a considerable loss of appetite occurs only a few days before death. By observing these precautions the control rats were kept in a healthy condition throughout the experiment, in spite of the adjustment of the consumption of the diet to that eaten by the vitamin A-deficient rats.

The paste, as in previous metabolism experiments, was given in a semi-liquid form in order to prevent the rats from dropping the food inside the cage, and so into the urine and faeces containers. The composition of the paste was as follows:

	Parts by wt.
Heated caseinogen	18
Wheat starch	50
Cotton-seed oil (irradiated ½ hr.)	15
Salt mixture	5
Dried yeast	18·3
Lemon juice	5
Water	181·7

The cotton-seed oil in the amount used in the diet was considered to be a sufficient source of vitamin E [Evans and Burr, 1927]. The control rats always ate their ration completely.

For the estimation of the general absorption from the alimentary canal, the absorption of nitrogenous substances (*i.e.* mainly proteins) and of fat was studied, since the digestion and absorption of these substances are more complicated processes than in the case of carbohydrates or salts and are, therefore, a clearer indication of the state of absorption in general. The estimation of nitrogen in the food, food residues, urine and faeces was made by the Kjeldahl method, whilst for the estimation of fat the Holt, Courtney and Fales [1919] method was used. In this method the soaps of the dried material are first hydrolysed by aqueous HCl, and to this acid mixture is added an equal volume of 96 % alcohol. This is then extracted with ethyl ether and light petroleum in the cold in a special apparatus. In our experience this method of fat estimation gives satisfactory results and we wish to express our thanks to Dr G. A. Harrison for being so kind as to demonstrate the method to us.

Four day periods were chosen for nitrogen and fat estimations, such periods

being sufficiently long to overcome the difficulty of accurate separation of the urine and faeces of each period [Korenchevsky and Dennison, 1932]. However, in order to economise space, the results of the experiments (summarised in Tables I, II and III) are given as averages for 8 day periods, except in the last period, in which, because of the considerable loss of appetite, the periods for the two pairs of rats were 2 and 3 days respectively. At the end of this period the rats were killed, the pair, Nos. 1756 and 1758 on the 84th day of age and the pair, Nos. 1757 and 1759, on the 85th day of age, having been on the metabolism experiment 26 and 27 days respectively.

The influence of a vitamin A-deficient diet on the appetite and weight of the rats.

In Table I the figures in the columns "N intake" and "fat intake" show a slow decrease in the appetite of rats 1756 and 1757 kept on a vitamin A-deficient diet. A very considerable reduction was noted on the last day of the last period (IV), thus decreasing the figure for this period, which, although it lasted only 2 or 3 days, was calculated, for the sake of comparison, for a week. In the first three periods the control rats, Nos. 1758 and 1759, kept on a complete diet, received per 200 g. of body weight less food than those rats with which they were paired, *viz.* 1756 and 1757 respectively, as shown by the nitrogen and fat intake (Table I).

Table I. *The absorption of nitrogen and of fat from the alimentary canal of the paired rats 1756 and 1758, 1757 and 1759 per week, per 200 g. of body weight.*

		Average wt. of	Intake		Urine	Faeces			N	Fat
No. of rat	No. of period	rat g.	N g.	Fat g.	N g.	Wt. g.	N g.	Fat g.	balance g.	absorbed g.
1756 on −A diet	I	140	3·69	15·17	2·51	5·02	0·33	0·81	+0·85	14·36
	II	150	3·33	13·66	2·42	5·19	0·34	0·83	+0·57	12·83
	III	157	3·00	12·31	2·44	4·42	0·30	0·73	+0·26	11·57
	IV	150	1·36	5·59	1·73	2·88	0·20	0·56	−0·57	5·03
1758 on complete diet	I	170	3·34	13·71	2·12	4·86	0·30	0·73	+0·92	12·98
	II	191	2·79	11·47	1·81	4·01	0·27	0·70	+0·71	10·77
	III	212	2·54	10·44	1·80	3·33	0·23	0·53	+0·51	9·91
	IV	216	1·75	7·20	1·41	2·98	0·19	0·53	+0·15	6·67
1757 on −A diet	I	152	3·88	15·93	2·54	5·72	0·37	0·79	+0·97	15·14
	II	162	3·03	12·45	2·36	4·49	0·29	0·74	+0·38	11·71
	III	161	2·90	11·90	2·33	3·85	0·27	0·64	+0·33	11·26
	IV	157	2·19	9·00	2·14	4·06	0·28	0·74	−0·23	8·26
1759 on complete diet	I	163	3·84	15·79	2·22	6·18	0·40	0·87	+1·22	14·92
	II	185	2·89	11·85	2·01	4·34	0·31	0·71	+0·57	11·14
	III	195	2·63	10·79	1·89	3·35	0·24	0·59	+0·50	10·20
	IV	204	2·49	10·23	1·87	4·08	0·28	0·72	+0·34	9·51

Table III shows the changes in weight compared with the food intake from the beginning of the experiment to the end of the period III, *i.e.* during the first 24 days of the metabolism experiment when the appetite, although less, was still satisfactory. The figures for the last period IV are not included in this Table since in this period the difference in the amounts of food consumed by the deficient and normal rats was considerable. The figures in Table III

are the actual figures, and not the figures calculated per 200 g. of body weight as in Tables I and II.

Table II. *Percentage of the nitrogen intake excreted in the urine, deposited in, or lost from, the body and non-absorbed from the alimentary canal; also percentage of the fat intake non-absorbed.*

No. of rat	No. of period	% N faeces to N intake	% N urine to N intake	% N balance to N intake	% fat non-absorbed to fat intake
1756 on −A diet	I	8·94	67·9	+23·0	5·33
	II	10·21	72·7	+17·1	6·07
	III	10·00	81·3	+ 8·7	5·93
	IV	14·70	127·2	−41·9	10·02
1758 on complete diet	I	8·98	63·5	+27·5	5·32
	II	9·67	64·8	+25·4	6·10
	III	9·05	70·9	+20·1	5·08
	IV	10·85	80·6	+ 8·6	7·36
1757 on −A diet	I	9·53	65·4	+25·0	4·96
	II	9·56	77·9	+12·5	5·94
	III	9·30	80·3	+10·3	5·37
	IV	12·78	97·7	−10·5	8·22
1759 on complete diet	I	10·41	57·8	+31·8	5·50
	II	10·72	69·5	+19·7	5·90
	III	9·12	71·8	+19·0	5·47
	IV	11·24	75·1	+13·6	7·13

Since the control rats were growing more quickly than the vitamin A-deficient rats the amount of food per 200 g. of body weight given to and completely consumed by the rats on a complete diet was less than that consumed by the rats on the vitamin A-deficient diet.

It should also be noted that it was only possible to make an approximate, immediate, estimation of the food eaten on the previous day by the vitamin A-deficient rats, since this was calculated by subtracting the weight of the residue of the food left by the rat from the weight of food given. There is, however, always during the 24 hours, some evaporation of water from the paste left unconsumed.

In later experiments, which will be described in the next paper, the error due to this evaporation is, as far as possible, corrected. For our conclusions, however, it was fortunate that the control rats on a complete diet were allowed less food per 200 g. of body weight than was consumed by the rats on the vitamin A-deficient diet, and in spite of this grew much better. This is especially evident with the pair of rats Nos. 1757 and 1759, for while the rat on deficient diet stopped growing during the 16 days of periods II and III (Table I) and finally lost weight, rat 1759 continued to grow until it was killed. Certainly there is little doubt that the control rats would gain in weight more if the amount of food given were increased.

The rats kept on the vitamin A-deficient diet, however, do not seem to be able to increase in weight in normal correlation to the amount of food ingested. If, from the actual increase in body weight and the actual nitrogen intake of the four experimental rats the increase in body weight per g. of nitrogen intake is calculated, the above statement would seem to be corro-

borated (Table III). Thus, whilst 1 unit of vitamin A-deficient food, as measured by 1 g. of nitrogen, produced an increase in body weight of only about 2·5 g., the same amount of complete diet produced an increase in the weight of the two control rats of 6·54 and 5·55 g. respectively, *i.e.* the increase in weight in the control rats was on an average 143 % greater than in the deficient rats.

Table III. *Gain in body weight per g. of nitrogen intake during the experimental period of the first 24 days.*

No. of rat	Total gain in weight (actual) g.	N intake (actual) g.	Gain in weight per g. of N intake g.
1756 –A	21	8·47	2·48
1758 Nor.	61	9·31	6·54
1757 –A	22	8·80	2·50
1769 Nor.	53	9·54	5·55

From the above experiments it is obvious that:

(1) the body weight of rats kept on vitamin A-deficient diet did not increase in normal correlation to the amount of food ingested;

(2) during the periods when the growth of the rats is checked or remains stationary the decrease in the appetite, with the consequent decrease in food intake, of rats kept on a vitamin A-deficient diet could only partly explain the decrease in rate of growth, and as a chief cause is probably only applicable to the last stages of the deficiency.

Nitrogen and fat absorption.

The comparison of the figures for nitrogen and fat excreted in the faeces, *i.e.* non-absorbed, shows that absorption during all the periods except the last was slightly less in only one of the rats on the vitamin A-deficient diet, No. 1756, as compared with its paired control, No. 1758 (Table I). Only in the last period was the absorption considerably affected in both deficient rats. When absorption of nitrogen and fat is expressed as the percentage of the intake (Table II) it is evident that such a small decrease in the absorption cannot explain the difference in growth.

This conclusion is seen to be justified in view of the results obtained with the second pair of rats Nos. 1757 and 1759, there being no constant appreciable difference in absorption in these two rats during the first three periods (Tables I and II), whilst the difference in growth still occurred.

It is true that during the last (fourth) period (just before the rats were killed) the percentage of non-absorbed nitrogen and fat (Tables I and II) was more pronounced in the deficient than in the control rats. It must be mentioned, however, that in rat 1756 there was an inclination to diarrhoea, and only in this rat was the non-absorbed nitrogen raised to 14·7 % and the non-absorbed fat to 10·02 %. During this last period, close to death, the absorption of nitrogen and fat were decreased more noticeably but still not very greatly.

Taking all the above data into consideration the following statements can be made.

(1) Except for a period of a few days before being killed, the absorption of nitrogenous and fatty substances from the alimentary canal was very slightly, if at all, decreased in rats kept on the vitamin A-deficient diet.

(2) Therefore the checking or cessation of growth of rats on a vitamin A-deficient diet cannot be explained by a decrease in the function of absorption of food from the alimentary canal.

(3) Only during the last period before death, when the vitamin A-deficient rats have begun to lose weight, and also to have an inclination to diarrhoea, does any impairment of the function of absorption become apparent and even then not to a very great extent.

Nitrogen metabolism.

Since the rats were young, nitrogen deposition for growth should be considerable. In both the vitamin A-deficient rats, as compared with their paired controls, although the nitrogen intake per 200 g. of body weight was greater, the deposition of nitrogen in the body was less (Table I). The reduction in food intake also produced a greater reduction in the nitrogen deposition in the deficient rats than in the rats on the complete diet. Table II shows also that in the vitamin A-deficient rats the percentage of the nitrogen intake excreted in the urine was greater, while that deposited in the body was less than in their respective controls.

These facts indicate that from the beginning of the metabolism experiment, namely, from the 25th day of feeding with the vitamin A-deficient diet to the end of the experiment (*i.e.* for about 4 weeks), the nitrogen metabolism of these rats was increased, during some periods to a marked degree (compare the periods II, III and IV in Tables Nos. I and II).

Discussion.

From this observation on the loss of appetite and consequent decrease in food intake, and also in some instances a lessened absorption from the alimentary canal in rats kept on a vitamin A-deficient diet, it is possible to conclude that these factors alone cannot explain completely the checking of growth which was observed. In rats kept on a vitamin A-deficient diet, during the period preceding death, when the loss of appetite becomes considerable, the influence of the decreased food intake on the rate of growth seems to become the important, and probably, the chief factor. Even in this period, however, and more particularly in the previous periods, the increased nitrogenous metabolism (*i.e.* chiefly protein metabolism), may be one of the factors which check the growth of the vitamin A-deficient rats, since the deposition of nitrogen has already been decreased by a lessened food intake. Certainly with a normal appetite such a degree of increase of metabolism as was observed in the experiment would hardly alone be able to check growth. This patho-

logical condition of metabolism may be the direct result of deficiency of vitamin A, or the indirect result of the deficiency on the endocrine organs controlling metabolism. The data at present available give no indication as to which of these factors is responsible.

The chief factor in checking the growth seems to be that one unit of the vitamin A-deficient food consumed by the rats produces a much smaller increase in body weight than the same unit of complete diet. Perhaps this fact is correlated with the increased metabolism. If even a slight pathological condition of absorption is also present the total effect on growth will be greater.

Summary.

1. The body weight, appetite, intake and absorption of fat and nitrogen, and the nitrogenous metabolism of two male rats kept on a vitamin A-deficient diet were compared with those of two control litter-mates kept on a complete diet.

2. Each control rat was paired as regards food intake and the other conditions of the experiment with a rat on a vitamin A-deficient diet.

3. During the period of checking or cessation of growth of the rats the decrease in food intake was only moderate and could offer only a partial explanation of the change in rate of growth, since the amount of food, if calculated per 200 g. of body weight, consumed by the rats on the deficient diet was even larger than that consumed by the rats on the complete diet.

4. During the same period the absorption of nitrogen and of fat was not appreciably altered and could not, therefore, be the factor responsible for this checking of growth.

5. In the last period, when the appetite was greatly decreased, the decrease in food intake was probably the main cause of the loss of weight, this effect being augmented by the definitely decreased absorption from the alimentary canal observed during the last period.

6. During the whole period of observation the nitrogenous metabolism increased.

7. This increase of metabolism occurring simultaneously with the decreased food intake may check the normal growth of rats kept on a vitamin A-deficient diet.

8. In such rats the increase in body weight was less than in rats kept on a complete diet when correlated with the same unit of food ingested. This fact would seem to be the chief factor in checking the growth of rats kept on a vitamin A-deficient diet.

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CLVIII. THE INFLUENCE OF VITAMIN A DEFICIENCY ON MALE RATS IN PAIRED FEEDING EXPERIMENTS.

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The object of this investigation was to determine whether the changes produced in body weight and in the deposition of fat in male rats on vitamin A-deficient diets are due to this deficiency alone or to the accompanying reduced appetite; and also to study the changes in weight of the sexual and endocrine organs.

The following experiments, therefore, supplement those published in a previous preliminary paper [Sampson, Dennison and Korenchevsky, 1932].

In the preliminary paper observations were made on 2 rats on vitamin A-deficient diet and on 2 control rats on a complete diet, paired in regard to food intake and other conditions with the respective rats on the vitamin A-deficient diet.

In spite of the decreased appetite during the period of cessation of growth the rats on vitamin A-deficient diet (– A diet) consumed a larger amount of food per 200 g. of body weight than did those on the complete diet. At the same time one unit of the vitamin A-deficient food consumed by the rats produced a much smaller increase in body weight than the same unit of complete diet. The latter fact was perhaps correlated with the simultaneously increased nitrogen metabolism which was observed. It was suggested that these facts give the chief explanation of the checking of growth produced by the direct deficiency of vitamin A irrespective of appetite since, during this period, the absorption from the alimentary canal did not show any significant changes.

We were unable to find in the literature any record of the use of the paired-feeding method in investigations of vitamin A deficiency, or, consequently, any accurate estimations by weight of changes in fat deposition, or in sexual or endocrine organs due directly to vitamin A deficiency.

It is known that, in general, fat deposition is decreased in animals kept on a vitamin A-deficient diet. It is also known that, in such animals, the actual weight of the testes is less than in those on unlimited complete diet. Histological examination reveals degeneration of the seminiferous tissue [see references by Mason, 1930].

A critical review of these references will be given in a subsequent paper elsewhere, when dealing with the histological changes observed in the sexual and endocrine organs of the rats on which the experiments described in this paper were performed.

Technique.

The experiments were performed on 63 male rats belonging to 12 litters. They were reared on the stock diet used at the Lister Institute, described by Smith and Chick [1926]. The litters were placed on the experimental diets at the following ages:

Litters 6 and 7 on the 7th day.
Litters 1, 2, 3, 5, 10, 11 and 12 on the 21st to 26th day.
Litters 4, 8, 9 on the 27th to 33rd day.

The duration of the experiment was:

Litter 8—24 days.
Litters 1, 2, 3, 6, 7 (except rats 30 and 32, 28 days)—9 days.
Litters 10, 12—31 to 35 days.
Litters 5, 11—39 to 42 days.
Litter 4—52 days.

The final ages and the weights of the rats are given in Table III.

In Exps. I and II, the diet was given *ad libitum* to all the rats. In the remaining ten experiments the rats were paired. One member of each pair received a diet deficient in vitamin A only, the other member a diet adequate in all respects. On the vitamin A-deficient diet the food intake gradually decreases. This may affect not only the body weight but also the weight of the organs and their structure and function. To discriminate between the effect of decreased food intake and that of the vitamin A deficiency, it is necessary that the paired control animal should receive the adequate diet in the same quantity as was consumed by the litter mate on the deficient diet. This procedure, adopted in this and the previous investigations [Sampson *et al.*, 1932] did not necessitate any considerable reduction in the food intake of the control rats since the pairs were killed on the day on which the vitamin A-deficient litter-mate showed a marked loss of appetite, which sometimes also resulted in a loss of body weight. Owing to these precautions the paired control rats were kept in good health throughout the whole experiment. In order to make a comparison, in some experiments a third litter-mate was allowed the complete diet *ad libitum*.

The period required for exhaustion of the vitamin A reserves in the young rats may be shortened by substituting a vitamin A-deficient diet for the usual diet at the 17th day of lactation—the time when the young begin to consume the stock food directly. Of course, the mother is also consuming this deficient diet from this period. By this method, as was shown by Korenchevsky [1922], the effects of the deficiency may be accentuated. In an endeavour to obtain

extreme changes the mothers and the young of the litters Nos. 6 and 7 were placed on a vitamin A-deficient diet from the 17th day of lactation.

The composition of the – A diet was as follows: caseinogen (heated) 180, starch (wheat) 500, cotton-seed oil (irradiated) 150, salt mixture 50, yeast (dried) 183, lemon juice 50, water 700 parts by weight.

The control animals received the same diet supplemented with daily doses of 20 to 100 mg. (according to the weight of the animal) of cod-liver oil (Fairbank Kirby). To keep the caloric intake of the paired litter-mates equal, the rats on vitamin A-deficient diet received an equal amount of hydrogenated cotton-seed oil.

In Exps. I and II, however, the control animals received a paste in which the cotton-seed oil was replaced by 120 parts of butter and 30 parts of cod-liver oil. All the rats of these two litters also received 6 drops of wheat-germ oil daily to supply an extra amount of vitamin E and a small though inadequate amount of vitamin A, so that the vitamin A deficiency in litters 1 and 2 was much less complete than in litters 3 to 12. Vitamin E was supplied in the latter experiments by hydrogenated cotton-seed oil, since according to Evans and Burr [1927] this fat, when fed in the amount given above, is a satisfactory and sufficient source of the antisterility vitamin.

The diet in all the experiments, except IX, was given in the form of a paste. In Exp. IX the rats were given a diet of the same composition but without the addition of water. It was hoped that a more accurate weighing of the dry residue would thus be obtained. Unfortunately the vitamin A-deficient rats during the period of pronounced deficiency scattered this dry diet badly and the collection of the residue was much more difficult than with the paste.

At the same time the accurate estimation of food eaten by the deficient rats was of the utmost importance since this amount determined the amount of diet to be given to the paired control on the following day.

The chief difficulty was to determine the amount of the residue of the paste since, by the end of 24 hours when the residue was collected, some of the water in the paste had evaporated.

In order to prevent this evaporation as much as possible and also to prevent the scattering of the food the paste was firmly pressed into heavy metal cups. Though helpful, this precaution was insufficient, so that it was necessary to introduce a correction for the evaporation of water.

The amount of evaporation of water from the diet was determined in the following way. Weighed samples of the paste in quantities equal to the amount of food given to the rats and to the amount of residue usually left were placed on the same shelves as those on which the rat-cages were kept. At the end of 24 hours the samples were reweighed and the amount of water evaporated estimated. At frequent periods during the investigation observations of the evaporation were made and on the average the loss of water was found to be nearly constant, probably owing to the fact that the room temperature was kept practically constant.

Table I. *Average evaporation of water from the food residue at the end of 24 hours.*

Weight of sample g.	Loss of water by evaporation g.
1 to 2	0·5
2 to 5	1·0
5 to 10	2·0

The loss of water was on the average as given in Table I.

These figures indicate that to the weight of paste uneaten at the end of 24 hours and partially dried, from 0·5 to 2 g. must be added according to the weight of the residue to offset the evaporation of water.

In order to estimate the accuracy of this method of correction for evaporation the data of the food consumption obtained in this way were compared with the food consumption calculated on the basis of the nitrogen intake in the experiment described in the previous paper [Sampson, Dennison and Korenchevsky, 1932]. The results are summarised in Table II.

Table II. *The relation of the quantity of paste consumed, estimated by weighing the paste and residue and correcting for evaporation, to 1 g. of the actual intake of nitrogen, for the different periods of the experiments on rats 1756 and 1757.*

Period		Corrected paste intake	Nitrogen intake by analysis	Relation of paste intake to 1 g. N intake
1756	I	127	2·70	47·0
	II	113	2·46	45·9
	III	110	2·45	44·9
	IV	122	2·52	48·4
	V	120	2·51	47·8
	VI	105	2·19	47·9
	VII	46	1·02	45·1
1757	I	138	2·93	47·1
	II	137	2·93	46·7
	III	116	2·51	46·2
	IV	114	2·38	47·9
	V	100	2·12	47·2
	VI	120	2·54	47·2
	VII	78	1·72	45·3
			Average	46·7

If the method were perfect 1 g. of nitrogen would always have corresponded exactly to the same amount of paste, for which the nitrogen content was found, by analysis, to have been constant throughout the experiment. Fluctuations, however, were observed, the lowest figure being 44·9 and the highest 48·4, with an average of 46·7. Since the maximum deviation from the average did not exceed 3·9 % this method of correction may be considered to be quite satisfactory.

To the rats mentioned in Table II the paste was given in a semi-liquid form, while to all other rats it was given in the form of a dough-like paste. In these latter cases, therefore, some scattering of the food always occurred and consequently the possible error was greater. Great care was taken,

however, to collect accurately the scattered food and the scattering was considerably reduced by pressing the ration firmly into the metal cups to which it adhered quite well.

For the interpretation of the changes in the thyroid the consideration of the amount of iodine present in the diet is of importance. The vitamin A-deficient diet used differed in iodine content from the complete diet since the cod-liver oil given to the rats on the complete diet contains iodine, while cotton-seed oil does not. Therefore some of the rats of litters 10, 11 and 12 (marked with an asterisk, Table III) were given 7 and 30 γ of iodine per day dissolved in cotton-seed oil. Both these doses, in any case, are larger than the maximum amount of iodine in the daily quantity of cod-liver oil given to the rats [see Orr and Leitch, 1929, p. 88].

All rats were weighed at least twice weekly, and examined for symptoms of vitamin A depletion. At the onset of xerophthalmia the rats were weighed daily. The experiments were continued for 24 to 52 days in each case until xerophthalmia was obvious or until loss of appetite led to slower gain in, or loss of, body weight. The rats were killed by coal gas, pairs being killed simultaneously.

For dissecting and weighing the organs the technique proposed by Korenchevsky [1930; 1932] was used. Thus the intra-abdominal fat and penis were weighed fresh, while the testes, prostate with seminal vesicles, thyroids, adrenals, pituitary and thymus glands were weighed after remaining in Allen's modification of Bouin's fixative for 48 hours. The fixed material was subsequently used for histological study.

Experimental results.

Body weight and appetite.

The age and the initial and final weights of the rats are given in Table III. The abbreviations for the diets which are employed in the tables are also used in the text (see footnote to Table III). Since the $-$A diet used in the experiments contains a large amount of vitamin B in the form of dried yeast, the rats on this diet suddenly lose their appetite, usually only a few days before death. As mentioned above, on the day when a considerable loss of appetite and of body weight occurred the pairs were killed. The gain or loss in weight during the last day is given, to indicate the condition, *i.e.* retarded growth, cessation of growth or fall in weight at this time.

It should be noted that of the 34 rats on the $-$ A diet, loss of weight, generally small, occurred in 19 rats only, whilst xerophthalmia was noted in all except the rats of litter 1. The initial weights of the rats of this litter on $-$A diet were greater than those of any other rats on this diet, and the gain in weight was continuous up to the day of killing. This shows that at killing vitamin A reserves were still present in these rats.

Table III. *Body weight of rats in g.*

	CoU diet			CoP diet			− A diet				
		Weight			Weight			Weight			
Litter no.	Rat no.	Initial	Final	Rat no.	Initial	Final	Rat no.	Initial	Final	Gain or loss during last day	Final age in days
1	1	58	289	—	—	—	3	56	259	+ 4	62
	2	58	289	—	—	—	4	61	257	+ 3	62
2	5	40	231	—	—	—	8	38	185	− 5	58
	6	36	254	—	—	—	9	40	179	+ 3	58
	7	35	237	—	—	—	10	38	180	− 5	58
3	—	—	—	11	30	146	13	34	119	− 7	56
	—	—	—	12	31	147	14	35	130	+ 3	56
	—	—	—	—	—	—	15	33	119	+ 3	56
4	—	—	—	16	48	214	18	50	145	− 9	84
	—	—	—	17	48	205	19	48	149	−16	85
5	20	46	249	22	45	251	24	45	195	+ 3	68
	21	38	264	23	40	222	25	41	149	− 8	68
	—	—	—	—	—	—	26	43	180	− 3	68
6	—	—	—	27	38	123	28	38	121	− 1	55
7	—	—	—	29	38	150	31	40	130	+ 1	55
	—	—	—	30	35	87	32	35	68	− 7	49
	—	—	—	—	—	—	33	36	109	± 0	55
8	34	37	95	—	—	—	37	35	57	−10	51
	—	—	—	35	43	91	38	42	87	± 0	51
	—	—	—	36	35	78	39	39	73	− 4	51
9	40	38	165	42	39	134	44	41	130	− 1	62
	41	39	165	43	41	148	45	43	130	− 4	59
10	—	—	—	46	42	145	49	51	142	+ 1	64
	—	—	—	—	—	—	50	51	115	−10	61
	—	—	—	47	48	152	51*	48	135	− 3	64
	—	—	—	48	44	133	52*	48	112	−11	61
11	—	—	—	53	50	185	55	50	155	− 9	67
	—	—	—	—	—	—	56	47	164	+ 2	67
	—	—	—	54	43	190	57*	52	165	± 0	67
	—	—	—	—	—	—	58*	49	147	− 5	67
12	—	—	—	—	—	—	60	55	123	− 1	63
	—	—	—	—	—	—	61	47	128	+ 2	63
	—	—	—	59	49	148	62*	49	122	+ 2	63
	—	—	—	—	—	—	63*	44	101	+ 3	63

Note. In the tables of this paper the following abbreviations are used: CoU = complete unlimited diet; CoP = complete paired diet, *i.e.* given in the same amount as is consumed by the paired rat on the vitamin A-deficient diet; − A = vitamin A-deficient diet.

In columns CoP and − A the numbers in the same line indicate paired animals.

* These rats received iodine.

Since both in litter 1 and litter 2 rats on the deficient diet received small amounts of vitamin A in the wheat-germ oil, these two litters are considered separately from the other 10 litters in all our averages and interpretations.

The weight and the appetite of the rats must be considered simultaneously. The actual food intake and the intake per 200 g. of body weight are given in Table IV. As can be seen from the figures in this table small discrepancies exist in most pairs between the amounts of food eaten by the rats of a pair. In Table IV in each of the litters in which the paired feeding method was used, *i.e.* litters 3 to 12, the figures of the paired − A-rat are placed below those of its paired control (CoP)-rat, *e.g.* in litter 3 in the column "Actual food

Table IV. *Daily average of food intake in g., actual and per 200 g. of body weight; and average daily gain of each rat per 10 g. of actual food intake.*

Litter no.	Diet	Actual food intake	Intake per 200 g. body weight	Gain per 10 g. of actual food intake
1	CoU	33·1, 30·4 — —	37, 34 — —	1·9, 2·1 — —
	−A	27·1, 25·1 — —	34, 31 — —	2·0, 2·1 — —
2	CoU	26·8, 27·9, 26·0 —	42, 41, 39 —	2·0, 2·2, 2·1 —
	−A	22·3, 20·4, 20·7 —	39, 36, 34 —	1·7, 1·8, 1·8 —
3	CoP	16·8, 16·2 — —	39, 37 — —	2·0, 2·0 — —
	−A	16·0, 16·5, 14·6 —	38, 40, 40 —	1·5, 1·6, 1·7 —
4	CoP	17·6, 17·6 — —	26, 27 — —	1·8, 1·7 — —
	−A	16·3, 17·7 — —	27, 29 — —	1·1, 1·1 — —
5	CoU	24·1, 26·4 — —	35, 39 — —	2·0, 2·0 — —
	CoP	23·7, 21·4 — —	36, 34 — —	2·1, 2·0 — —
	−A	23·6, 20·2, 22·6 —	37, 37, 37 —	1·5, 1·3, 1·4 —
6	CoP	11·5 — — —	32 — — —	2·2 — — —
	−A	16·9 — — —	43 — — —	1·4 — — —
7	CoP	16·3, 10·4 — —	37, 37 — —	2·0, 1·8 — —
	−A	17·0, 10·1, 14·3 —	40, 36, 37 —	1·6, 1·2, 1·5 —
8	CoU	12·6 — — —	41 — — —	1·9 — — —
	CoP	12·1, 11·4 — —	34, 36 — —	1·7, 1·6 — —
	−A	12·2, 11·5, 10·6 —	36, 36, 36 —	1·5, 1·2, 0·9 —
9	CoU	19·2, 20·5 — —	40, 41 — —	1·9, 2·0 — —
	CoP	15·7, 16·6 — —	38, 38 — —	1·8, 2·1 — —
	−A	16·5, 18·3 — —	37, 39 — —	1·6, 1·5 — —
10	CoP	16·0, — 17·2, 16·5	34, — 32, 35	1·8, — 1·7, 1·7
	−A	17·4, 16·6, 17·1, 15·6	35, 32, 32, 33	1·5, 1·2, 1·5, 1·3
11	CoP	19·7, — 19·8, —	32, — 33, —	1·8, — 1·9, —
	−A	20·1, 20·2, 21·0, 18·8	34, 36, 36, 33	1·3, 1·5, 1·4, 1·3
12	CoP	— — 15·0, —	— — 32 —	— — 1·9 —
	−A	15·7, 15·7, 14·7, 12·7	33, 36, 34, 35	1·2, 1·5, 1·4, 1·3

Note. Each figure relates to a rat in the order of the respective numbers of the rats, which can be ascertained by reference to the corresponding litters and groups in Table III.

intake" the figures for the respective pairs are 16·8 and 16·0; 16·2 and 16·5; in the next column they are 39 and 38; 37 and 40 and so on.

When there is a slight excess in the amount of food eaten by the control paired rats as compared with that eaten by the −A rats it is chiefly due to differences in intake on the first and last days of the experiment. On the first day of the experiment the control paired rat may eat more than the deficient rat since actual pairing does not start till the second day when the control rat receives the same amount as was eaten the previous day by the −A rat. On the last day of the experiment, in most cases, the food was completely consumed by the control rats, while the deficient rats might eat very little.

When the amount of food eaten by the control rats is slightly less than that eaten by the −A rats, it is due, in some cases, to giving a little less food to the CoP rats. This was done in order to be certain that the control rats did not receive more than the experimental rats and in order to increase the effect of limitation of the diet. Care was taken, however, not to injure the healthy condition of the control rats by such adjustments. In other cases

the smaller intake of the CoP rats was due to their refusal to consume the diet completely. As is well known some rats dislike synthetic diets. A consideration of these CoP rats on reduced intake as compared with their pairs is of especial importance in our conclusions.

Of those rats receiving the complete unlimited diet, the body weight, in about half of the individuals, was only slightly greater than that of the rats on a complete but paired diet (Table III, litters 5, 8 and 9). This shows that, because of the precautions mentioned above, the limitation of the diet of the paired rats was very small (see Table IV), except in those cases in which the rats disliked the diet and, therefore, left part of it unconsumed.

A comparison of the paired rats reveals that the gain in weight of the rats on CoP diet is greater than that of the rats on −A diet. In a few cases, rats 27, 35, 36 and 42, this difference is small, from 2 to 9 g., but in all the other CoP rats it is much greater, being from 12 to 30 g. in 9 rats and from 31 to 74 g. in the remaining 6 rats. At the same time it can be seen from Table IV that in litters 3 to 12 the food intake of the rats on CoP diet was, in most cases, the same as that of the rats on −A diet or even less. This is evident in the figures for actual food intake but is accentuated when the intake is calculated per 200 g. of body weight. For litters 1 and 2, in which the vitamin A deficiency was only partial, the increase in weight produced by one unit of food complete or deficient in vitamin A was also calculated. The results obtained are given as "Gain per 10 g. of actual food intake," in the third column of Table IV. In all the rats which showed vitamin A deficiency, even in those of litter 2, the gain in weight produced by one unit of vitamin A-deficient diet was, without exception, less than that produced by the same unit of complete diet. In litter 1, in which, as was mentioned above, the depletion of vitamin A was incomplete, this difference in rate of growth per unit of food intake of −A as compared with CoP diet did not occur. The absence of this change in this litter only emphasises its presence in all the other litters. In litters 3 to 12 the actual daily average of food intake for the rats on CoP and deficient diets was 16·4 and 16·6 g. respectively; the average daily food intake per 200 g. of body weight was 34 and 36 g. respectively and the average daily gain in weight per 10 g. of actual food intake 1·9 and 1·4 g. respectively. It is worthy of note that the gain in weight per 10 g. of food intake is nearly the same in the rats on complete diet, in litters 5 and 9, both when consumed *ad libitum* (CoU diet) and when adjusted in quantity to that consumed by the deficient rats. In litter 8 the gain in weight on CoU diet was greater.

In litters 5, 8 and 9, in which all three groups are represented, the body weights in groups CoP and −A are on the average about 11 % and 26 % respectively less than in group CoU. In other words, roughly two-fifths of the deficiency in weight of −A rats in these experiments was due to decreased appetite and three-fifths was due to the direct influence of vitamin A deficiency.

These data also indicate that in order to obtain the same increase in body weight it is not sufficient to ingest the same unit of food. Only if vitamin A is present is this so, otherwise the food ingested in the same quantity will produce a smaller increase in body weight and presumably in the growth of the animal.

Fat deposition.

As judged by the average weights of retroperitoneal and testicular fat in litters 2 to 12, the weight of the fat was less in the −A rats than in those on complete diet (Table V). In the three litters 5, 8 and 9, in which all three

Table V. *Average weight of body fat of rats in each group of each litter (g.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	9·68	6·69	±0·69	—	—	—	7·75	6·01	±0·16
2	6·23	5·34	+0·25 −0·42	—	—	—	2·60	2·87	+0·26 −0·17
3	—	—	—	4·55	6·21	±0·43	2·92	4·77	+0·41 −0·79
4	—	—	—	8·64	8·20	±2·16	3·60	4·88	±1·41
5	9·18	7·17	±0·44	6·01	4·95	±2·02	3·24	3·65	+0·43 −0·85
6	—	—	—	2·96	4·80	±0	2·85	4·70	±0·00
7	—	—	—	1·97	3·11	±0·76	1·42	2·58	+0·82 −1·05
8	1·07	2·25	±0·00	0·77	1·78	±0·53	0·53	1·02	+0·26 −0·28
9	4·87	5·90	±0·09	2·53	3·54	±0·85	2·08	3·19	±0·27
10	—	—	—	3·53	4·91	+1·03 −0·54	1·84	2·94	+0·64 −0·68
11	—	—	—	7·33	7·81	±0·39	4·58	4·47	+1·48 −1·45
12	—	—	—	4·25	5·74	±0	1·84	3·07	+0·41 −0·78

groups are represented, the percentage deficiency of fat per 200 g. of body weight in the CoP group and in the −A group, in relation to the CoU group, and in the −A group in relation to the CoP group, was calculated.

It was found that on the average in these three litters (1) the fat deposition was decreased by about 31 % when the amount of complete diet was adjusted to that consumed by the −A rats; (2) it was decreased by about 26 % in the rats on vitamin A-deficient diet as compared with those which consumed, on an average, the same, or even slightly less, amounts of food rich in vitamin A; (3) the deficiency of fat in the −A group in relation to the CoU group was on the average about 49 %. In litter 2 this decrease was 46 %. Therefore, about three-fifths of the deficiency of fat deposition was due to the decrease in appetite and two-fifths to the direct influence of the vitamin A deficiency. In litters 3 to 12 the percentage deficiency of fat in the

rats in the −A group in relation to those in the CoP group was 23, 40, 26, 2, 17, 21, 40, 40, 33 and 47; on the average 29 %, which is nearly the same as the 26 % obtained with the three litters mentioned above. The unusually small deficiency of 2 % occurred in litter 6, in which the control rat in the CoP group ate much less than his litter-mate in the −A group (see Table IV).

Certainly the decrease of stores of fat in the body will vary with the period of time at which the −A rats are killed. If, however, as was the case in our experiments, the rats are killed in a more or less satisfactory nutritional condition, with xerophthalmia well developed, but a few days before or at the beginning of the marked fall in weight about three-fifths of the average deficiency will be due, at this period of vitamin A deficiency, to the decreased food intake, and about two-fifths of the deficiency to the direct influence of vitamin A deficiency irrespective of the appetite.

Testes.

In general the actual weight of the testes in −A groups was smaller than that of the rats on complete diet. In litters 5, 8 and 9 the weights of the testes per 200 g. of body weight (Table VI) were slightly greater (about

Table VI. *Average weight of testes of rats in each group of each litter* (*g.*).

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	3·00	2·08	±0·02	—	—	—	2·81	2·18	±0·07
2	2·66	2·21	+0·06 −0·04	—	—	—	2·31	2·55	+0·43 −0·41
3	—	—	—	1·97	2·68	±0·09	1·95	3·13	+0·13 −0·05
4	—	—	—	2·30	2·20	±0·03	1·86	2·53	±0·06
5	2·42	1·89	±0·10	2·55	2·17	±0·19	1·78	2·00	+0·42 −0·50
6	—	—	—	1·69	2·75	±0·00	1·17	1·94	±0·00
7	—	—	—	1·55	2·57	±0·18	1·32	2·90	+0·54 −0·58
8	1·16	2·45	±0·00	1·17	2·79	±0·26	1·05	2·91	+0·20 −0·12
9	1·84	2·23	±0·02	1·73	2·45	±0·01	1·76	2·70	±0·18
10	—	—	—	1·80	2·52	+0·10 −0·13	1·67	2·68	+0·49 −0·33
11	—	—	—	2·54	2·71	±0·10	1·97	2·52	+0·88 −0·70
12	—	—	—	1·88	2·54	±0·00	1·36	2·30	+0·22 −0·34

13 %) in the group CoP than in the CoU group. In the −A group in relation to the same group CoU, the average increase was about the same, namely 15 %.

Although the actual weight of the testes of the rats on vitamin A-deficient diet was always less than that of the rats on CoP diet, when calculated per

200 g. of body weight, the change was not constant. There was an increase of 6 to 17 % in 6 litters, a decrease of 7 to 9 % in 3 litters, of 30 % in litter 6. Taking into consideration the fact that in litters 1 and 2 there was also an increase of 5 % and 15 %, and that, therefore, in most litters the testes of rats on vitamin A-deficient diet weighed more per 200 g. of body weight than those on CoP diet, it must be admitted that, as judged by the weight of the organs, in relation to the same unit of body weight, vitamin A deficiency seems to indicate hypertrophic rather than atrophic changes in the testes. This was not expected since, histologically, in all the testes of the above rats on vitamin A-deficient diet, degenerative or atrophic changes were found. No degeneration was found in the testes of the rats on the paired complete diet. These results will be communicated in detail later. An explanation, however, may be found in the fact that macroscopically these testes appeared to be oedematous, being soft and semi-transparent as compared with the testes of the control rats which were of firm consistency and of milky colour. Oedema of the testes would explain their greater weight.

Penis.

Table VII shows that in all the litters except 1 and 6, the penis, per 200 g. of body weight, was on the average about 21 and 24 % heavier in rats on −A diet than in rats on CoU or CoP diets respectively. In litter 1 there was practically no change, while in litter 6 the penis was smaller in the −A rats.

Table VII. *Average weight of penis of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	267	185	+25	—	—	—	283	180	± 3
2	219	182	± 1	—	—	—	199	219	+ 9 − 7
3	—	—	—	160	218	± 1	162	263	+48 −45
4	—	—	—	180	172	± 9	191	260	±10
5	224	175	± 4	210	178	±11	184	214	+28 −19
6	—	—	—	142	230	± 0	117	193	± 0
7	—	—	—	98	166	± 1	104	208	+33 −28
8	70	147	± 0	64	151	± 0	67	187	+17 − 9
9	160	194	±24	131	187	±13	143	219	±12
10	—	—	—	160	230	±20	165	260	±20
11	—	—	—	170	180	± 1	175	220	+20 −10
12	—	—	—	150	200	± 0	145	240	+20 −10

Litter 6 was exceptional, owing to the great difference in food intake between the control and −A rats (see Table IV) as was mentioned previously. The influence of vitamin A deficiency on the weight of the penis can be ascribed to the direct action of the deficiency since there was no significant change between the CoU and CoP groups (per 200 g. of body weight).

Prostate with seminal vesicles.

As can be seen from Table VIII decreased food intake caused a decrease in weight of the prostate with seminal vesicles, both in the actual figures and per 200 g. of body weight (compare litters 5, 8 and 9, CoU and CoP groups). Per 200 g. of body weight, on the average, this decrease was about 19 %.

Table VIII. *Average weight of prostate and seminal vesicles of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	1129	782	± 20	—	—	—	1189	922	± 22
2	888	738	+ 7 − 9	—	—	—	583	644	+113 −131
3	—	—	—	434	592	± 24	439	716	+243 −236
4	—	—	—	838	803	±129	887	1206	± 28
5	1257	975	±159	1066	906	± 72	878	1012	+ 99 − 52
6	—	—	—	269	438	± 0	262	434	± 0
7	—	—	—	216	346	± 71	203	388	+ 75 − 51
8	156	328	± 0	90	216	± 45	115	313	+ 32 − 50
9	561	680	±147	399	568	± 34	581	889	±166
10	—	—	—	457	635	+112 − 86	519	827	+ 84 − 69
11	—	—	—	701	747	± 17	747	951	+129 −194
12	—	—	—	466	629	± 0	388	649	+138 − 94

A comparison of the figures calculated per 200 g. of body weight of the groups on CoP and −A diets shows that the direct influence of vitamin A deficiency was towards an increase in weight of these organs, on the average by 32 % (with a maximum of 57 % and a minimum of 12 %). This influence was absent only in litter 6. Since the influence of decreased food intake is opposed to that of vitamin A deficiency the influence on the weight of these organs would probably depend on the relative action of these two factors.

Adrenals.

No influence of decreased appetite or of vitamin A deficiency on the adrenals could be observed from the results obtained (Table IX) when the

Table IX. *Average weight of adrenals of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	42·0	29·0	±0·3	—	—	—	42·4	32·8	± 1·8
2	39·9	33·3	±3·8	—	—	—	31·2	34·4	+ 3·6 − 2·2
3	—	—	—	44·3	60·1	±2·0	39·7	65·1	+22·6 −11·6
4	—	—	—	48·3	46·1	±2·6	41·9	57·1	± 5·7
5	46·9	36·5	±0·8	48·1	40·6	±0·3	37·1	43·2	+ 8·6 − 7·8
6	—	—	—	31·6	51·4	±0·0	28·1	46·4	± 0·0
7	—	—	—	44·2	76·3	±6·2	34·8	70·7	+14·6 −10·6
8	33·6	70·7	±0·0	30·9	73·2	±1·6	26·3	75·8	+27·3 −19·3
9	42·5	51·5	±1·8	40·7	58·0	±3·9	29·3	45·4	± 0·2
10	—	—	—	42·9	60·1	+7·0 −6·9	31·6	50·4	+ 7·0 − 3·7
11	—	—	—	46·5	49·6	±1·0	38·2	45·9	+ 3·1 − 2·5
12	—	—	—	44·0	59·4	±0	29·4	49·0	+ 3·7 − 3·8

weights were calculated per 200 g. of body weight; the glands weighing, on the average, 57·5 mg. in the CoP group and 54·9 mg. in the −A group. The actual weights in all the litters except litter 1 were smaller in the −A group.

Thyroids.

A comparison of groups CoP and CoU in Table XI shows that decreased food intake produced in most of the rats of litters 5, 8 and 9 a more or less pronounced decrease in weight of the thyroids, both in the actual figures (on the average, 22 %) and when calculated per 200 g. of body weight (on the average 13 %). On −A diet the weights of the thyroids, in most cases in the actual figures, but always when calculated per 200 g. of body weight, were greater than those of the controls, on an average by 43 %.

This diet did not include iodine. Iodine was given to a pair of rats in each of the litters 10 and 12, in the daily amount of 7 γ, and to one pair of litter 11 in the daily amount of 30 γ. As can be seen from Table X, in these three litters the thyroids of the −A groups again weighed more than those of the controls when no supplementary iodine was given, but the same or even less when iodine was added to the diet.

Table X. *Average weight of thyroids of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet			
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.		Iodine
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	content
1	28·4	19·7	±0·9	—	—	—	31·6	24·5	±1·3	−
2	25·3	20·9	+1·2 −1·0	—	—	—	25·0	27·5	+1·9 −1·6	− −
3	—	—	—	18·2	24·8	±2·4	21·6	35·1	+2·7 −1·5	− −
4	—	—	—	25·2	24·0	±0·6	24·8	33·7	±0·9	−
5	32·0	24·9	±1·5	24·5	20·8	±0·9	29·6	34·3	+4·7 −5·3	− −
6	—	—	—	13·0	21·1	±0·0	25·6	42·3	±0·0	−
7	—	—	—	15·4	26·2	±0·6	16·3	30·9	+5·0 −6·2	− −
8	16·0	33·6	±0·0	11·6	27·0	±1·0	16·0	43·9	+4·8 −6·1	− −
9	25·3	30·6	±5·0	20·9	29·7	±3·4	26·9	41·1	±7·7	−
10	—	—	—	19·9	27·7	+4·0 −2·2	23·0	36·0	±2·8	−
							17·5	28·6	±2·1	+
11	—	—	—	21·5	22·8	±2·3	22·7	28·5	±0·9	−
				—	—	—	19·0	24·4	±0·9	+
12	—	—	—	22·2	30·0	±0·0	20·1	32·6	±1·8	−
				—	—	—	14·4	23·4	±2·6	+

This indicates that the hypertrophy of the thyroids observed on our basal −A diet was produced, not by vitamin A deficiency, but by iodine deficiency. The control rats on CoU and CoP diets received sufficient iodine in the cod-liver oil which was given, this being one of the foods richest in iodine. Cotton-seed oil does not contain iodine in an amount detectable by micro-analysis.

Hypophysis.

In nearly all the litters, as can be seen from Table XI, the actual weight of the hypophysis was less in the −A and CoP rats than in those on complete unlimited diet. When, however, these weights are calculated per 200 g. of body weight, no significant or constant changes are observed, the weights being on the average 9·2 mg. and 8·7 mg. in the CoP and −A groups respectively.

Thymus.

No significant or constant changes were obtained in the weight of the thymus per 200 g. of body weight as a result of decrease in food intake in these experiments (Table XII).

In the rats on −A diet as compared with those on the CoP diet the weight of thymus per 200 g. of body weight in two (litters 8 and 12) of the 9 litters was not appreciably changed, whilst in the remaining seven it was less, on the average, by 23 % (maximum 38 %, minimum 12 %). The atrophic changes of the thymus in our experiments seem, therefore, to be related to the direct influence of vitamin A deficiency.

Table XI. *Average weight of pituitary of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	10·5	7·3	±0·5	—	—	—	9·6	7·5	±0·4
2	8·3	6·9	+0·4 −0·8	—	—	—	6·7	7·4	+0·2 −0·5
3	—	—	—	6·7	9·1	±0·4	5·8	9·4	+0·4 −0·7
4	—	—	—	7·2	6·9	±0·6	5·4	7·3	±0·4
5	9·1	7·0	±0·4	8·7	7·3	±0·7	6·3	7·2	+1·2 −1·3
6	—	—	—	5·2	8·4	±0·0	4·8	7·9	±0·0
7	—	—	—	6·6	13·0	±1·9	5·3	11·6	+7·2 −4·3
8	5·2	10·9	±0·0	5·0	12·0	±1·8	3·0	8·3	±0·1
9	6·8	8·3	±0·2	6·0	8·4	±0·2	7·1	10·9	±0·7
10	—	—	—	6·3	8·8	+0·2 −0·1	5·0	8·1	+1·2 −1·6
11	—	—	—	8·0	8·5	±0·1	6·1	7·7	+0·6 −1·2
12	—	—	—	6·8	9·2	±0·0	5·1	8·6	+1·4 −0·7

Table XII. *Average weight of thymus of rats in each group of litters nos. 4 to 12 (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—
4	—	—	—	317	302	± 8	182	249	± 76
5	580	453	±16	478	488	± 5	278	340	± 15
6	—	—	—	283	459	± 0	223	368	± 0
7	—	—	—	351	606	±51	200	374	+ 69 −105
8	226	475	± 0	160	378	±22	134	364	+ 54 − 49
9	377	457	±29	372	528	±13	235	360	± 44
10	—	—	—	159	225	+24 −41	125	198	+ 23 − 21
11	—	—	—	370	394	±26	268	341	+ 78 −123
12	—	—	—	227	307	± 0	184	309	+ 52 − 40

Iodine in −A diet.

We have already discussed the effect of addition of iodine to the −A diet on changes in the thyroid gland. No appreciable differences were noted, when iodine was included in the −A diet, in the changes above described in the other endocrine organs. Therefore, only in the case of the thyroid gland (Table X) in litters 10, 11 and 12 are the figures given separately for the groups fed on −A diet with and without the addition of iodine.

No influence was noted on the changes in body weight, gain per unit of food intake, or in fat deposition, or sexual organs.

Discussion.

It is clear from the data given above that, in rats kept on a vitamin A-deficient diet, two main factors are operating in influencing the organs and their functions.

The first is the decreased appetite, which, although directly caused by vitamin A deficiency, is in itself the cause of several secondary changes, which are entirely due to the effect of the decreased food intake.

The second factor is some other direct influence of vitamin A deficiency on the organs and their functions. In the animals kept on vitamin A-deficient diet the decreased food intake seemed to produce greater changes in fat deposition than in general body weight. For, while in our rats roughly 2/5 of the deficient gain in body weight was due to the decreased food intake, about 3/5 was caused by some other direct influence of the deficiency. In the fat deposition on the other hand these proportions were reversed, decreased food intake causing 3/5 of the deficiency in the body fat. This observation is in agreement with the general principle that with decreased food intake chiefly the carbohydrate and fat stores are affected first.

Measurements of body weight do not always represent the changes in growth of the animals, or the same degree of growth. In our young growing animals, however, these changes in weight roughly indicate the changes in growth. Thus in the following, with certain reservations, what is said about body weight also applies to the growth of the animals.

That the gain in weight is influenced to a considerable degree by the direct action of the deficiency is shown in all the litters in which vitamin A was depleted (*i.e.* litters 2 to 12). In every case one unit of the deficient diet produced a much smaller increase in body weight than the same unit of diet rich in vitamin A.

This property of vitamin A relating to building of tissue can be defined as an "anabolic principle." An increase in body weight and in growth of the animals is produced by this and by "the appetite producing" principle of this vitamin. In the absence of this "anabolic" principle, as is indicated by the results of our previous papers, the food consumed and absorbed is not deposited in the body normally or to a normal extent. The increased nitrogen

metabolism also testifies to this inability of the vitamin A-deficient organism to utilise the food normally for building new tissue.

In most cases the organs investigated were also under a twofold influence: that of the "appetite producing principle," *i.e.* of decreased food intake, and that of some other direct action of vitamin A deficiency.

With the sexual organs the unexpected result was noted of an increased weight, per 200 g. of body weight, of testes and notably of penis and prostate with seminal vesicles in most of the rats on −A diet as compared with those of the rats on CoP diet. In the case of the testes the oedematous condition would explain this increase, for histologically degenerative and atrophic changes were found in the seminiferous tissue which were completely absent from the testes of rats on CoP diet. In any case the changes in the testes did not affect the weights of the prostate with seminal vesicles or of the penis.

The decrease in food intake did not produce any significant changes in the weight (per 200 g. of body weight) of the penis, decreased the weight of the prostate with seminal vesicles and in some cases slightly increased the weight of the testes.

The thyroids in rats on −A diet were found to be hypertrophied. The −A diet, used by us, however, was deficient in iodine. When, in three litters, iodine was added to the −A diet no such hypertrophy was obtained. This change was therefore due to the iodine, rather than to the vitamin A, deficiency. In some rats decreased food intake on complete diet produced a more or less pronounced decrease in weight of the thyroids.

A direct influence of vitamin A deficiency on the thymus gland was evident as was shown by the decrease in weight of the gland.

No constant or significant changes were found in the adrenals and hypophysis.

Summary.

1. Using 63 male rats belonging to 12 litters a study was made of the influence of vitamin A deficiency on the body weight, fat deposition and the weight of the endocrine organs in order to distinguish the secondary changes produced by the decreased appetite from those produced by some direct influence of the deficiency.

2. Both these factors were found to be concerned in the changes produced in the organs and their functions.

3. At the stage of vitamin A depletion at which the rats were killed, about 2/5 of the deficiency in body weight would seem to be due to the decreased food intake, while 3/5 would seem to be caused by some other direct influence of the deficiency.

4. These proportions were reversed for the fat deposition.

5. In these experiments, in agreement with the previous results, it was found that one unit of the vitamin A-deficient diet produced a much smaller increase in body weight than was produced by the same unit of complete diet.

6. Therefore, vitamin A seems to have, in addition to an "appetite producing" principle, an "anabolic" principle, both of which are essential for building of the tissues, for increase in body weight and for growth of the animal.

7. When the weight of the organs is calculated per the same unit of body weight the following changes in the sexual organs were noticed. In most of the rats on vitamin A-deficient diet the weights of the testes and especially of the penis and of the prostate with seminal vesicles were greater than those of rats kept on a paired complete diet. The testes of the rats on the deficient diet seemed to be oedematous. The decreased food intake produced no noticeable effect on the penis, decreased the weight of the prostate with seminal vesicles and in some cases slightly increased the weight of the testes.

8. Reduction in the weight of the thymus was observed in most of the rats on the deficient diet and seemed to be due to the direct influence of vitamin A deficiency.

9. No significant changes were found in the weights of the adrenals or of the hypophysis.

10. The thyroids were hypertrophied only in the rats kept on the vitamin A-deficient diet which were used in the experiments. In rats receiving iodine in addition to this diet this change in the weight of the thyroids did not occur. It was, therefore, due to iodine deficiency.

11. No influence of iodine on any of the other changes caused by vitamin A deficiency was noticed.

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CLIX. THE INFLUENCE OF SUCCINIC, FUMARIC, MALIC AND ACETIC ACIDS ON THE DEPOSITION OF LIVER-GLYCOGEN.

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The formation of succinic acid from pyruvic but not from acetic acid in muscle perfusion experiments led Toenniessen and Brinkmann [1930] to suggest this acid as an intermediate product in the oxidation of sugar *in vivo*. In the organism the part of succinic acid as an intermediate substance in the possible transformation of fat to sugar has been suggested by Clutterbuck and Raper [1925] and as a possible degradation product of the unsaturated acids by Smedley-MacLean and Pearce [1931]. Information as to the fate of succinic acid in the animal body is therefore of importance.

In the phloridzinised dog, feeding with succinic and malic acids resulted in the excretion of extra sugar [Ringer, Frankel and Jonas, 1913; Cremer, 1913] whereas feeding with malonic acid gave a negative result. Although Geelmuyden [1920] obtained extra sugar when acetic acid was administered to a phloridzinised dog, his results have not been confirmed by other workers [Deuel and Milhorat, 1928; Ringer and Lusk, 1910]. Thunberg's [1920] suggestion that succinic is formed from acetic acid in the body is therefore improbable. We investigated the effect of adding the ammonium salts of the four acids, succinic, fumaric, malic and acetic separately to a diet rich in fat and poor in carbohydrate which was fed to normal rats for 3 days following 24 hours' starvation. The glycogen and fat contents of the livers were then determined. In some experiments, a quantity of glucose of equal calorific value replaced the above acids.

The control diet and that to which sugar had been added were much better taken by the rats than the diets to which the salts of the acids had been added. It will be seen from Table I that in many cases in which the percentage of glycogen was increased the diet consumed was considerably less than in the controls. Rats about 6 weeks old were used, those for each experiment being from the same litter. The controls received a basal diet consisting of:

Bran (washed to remove most of the carbohydrate) ...	1·9 g.
Agar-agar	0·7
Bloater paste	0·5
Butter	3·0
Moisture	8·6
Total per diem ...	14·7

The experimental rats were given each day 2 g. of the ammonium salt of one of the acids to be tested in addition to the basal diet. The sugar controls received in addition to the basal diet 1·38 g. glucose, this being approximately equicalorific to 2 g. of ammonium succinate. After three days the rats were killed by a blow on the back of the head. The liver was rapidly removed, sliced, divided into two portions, one for estimating glycogen and one for fat: each portion was weighed and put into boiling 60 % KOH. The glycogen was precipitated, hydrolysed and estimated following the method of Evans, Tsai and Young [1931] the glucose being determined by the Shaffer-Hartmann method [1920–21]. The KOH solutions for fat estimations were acidified with hydrochloric acid, completely extracted with light petroleum and ether and the residues weighed. In order more accurately to determine the I.V. of the fats in Exp. 2, the acidified KOH solutions were extracted with carbon tetrachloride in the manner described by Hynd and Rotter [1930]. The extracts were made up to a definite volume and portions were titrated with $N/30$ alcoholic KOH, an equal volume of alcohol being added to the carbon tetrachloride solution before each titration. The fat content was calculated assuming a mean molecular weight for the fatty acids of 280.

The details of the experiments are shown in Table II and the collected results in Table I. The average glycogen percentages group themselves fairly sharply into three classes: (1) the percentages in the rats fed with the control diet and with the control diet *plus* acetate were respectively 1·08 and 1·26; (2) in those fed with the control diet to which succinate, malate or fumarate had been added, respectively 2·27, 2·18 and 2·18; (3) in those fed with the control diet and sugar the average percentage was 3·5. The *dl*-malate gave somewhat higher figures than the *l*-form. Clutterbuck [1927] had found that succinic acid incubated with minced liver yielded 75 % *l*-malic and 25 % fumaric acid. The fact that in our experiments *dl*-malic acid led to a higher percentage of glycogen in the liver than the *l*-form was therefore unexpected.

The average percentage of fat in the livers was markedly higher in the controls but no significant differences between the percentages in the other groups could be detected: the figures for the group which had received the additional sugar and for that which had received the acetate were similar.

Conclusion.

These results furnish evidence that in the normal rat the addition of succinic, malic or fumaric acid to a diet poor in carbohydrate leads to an increase of glycogen in the liver. After the addition of acetic acid no significant increase in glycogen could be detected.

The feeding experiments were carried out by Miss E. M. Hume and Miss Henderson Smith, whose co-operation and help we gratefully acknowledge.

We desire to acknowledge a grant from the Department of Scientific and Industrial Research which made it possible to carry out this work.

Table I.

No. of exp.	Diet (basal) (wt. in g. per day)			% glycogen in the liver						% fat in the liver					
	Carbohydrate	Protein	Fat (butter)	Control	Acetate	Fumarate	Malate	Succinate	Sugar control	Control	Acetate	Fumarate	Malate	Succinate	Sugar control
1	0·910	0·327	3	1·31 ♀	0·89 ♀	—	—	—	—	—	—	—	—	—	—
				1·30 ♀	1·54 ♀	—	—	—	—	—	—	—	—	—	—
					1·74 ♀	—	—	—	—	—	—	—	—	—	—
2	—	—	—	1·18 ♀	2·55 ♂	—	—	2·46 ♂	—	*5·27 ♀	4·47 ♂	—	—	4·07 ♂	—
				0·95 ♂	—	—	—	—	—	7·43 ♂	—	—	—	—	—
				0·22 ♀	—	—	—	—	—	11·05 ♀	—	—	—	—	—
3	—	—	—	—	0·74 ♀	—	*l-* 1·72 ♀	1·49 ♀	2·11 ♀	—	6·89 ♀	—	*l-* 5·16 ♀	4·95 ♀	4·15 ♀
					1·09 ♀	—	*l-* 2·31 ♂	1·45 ♂	0·65 ♀	—	5·89 ♀	—	*l-* 3·50 ♂	3·31 ♂	4·68 ♀
4	0·937	0·326	3	—	2·00 ♂	—	*l-* 1·05 ♀	0·10 ♀	1·63 ♀	—	4·81 ♂	—	*l-* 5·69 ♀	7·98 ♀	9·29 ♀
					0·56 ♀	—	*l-* 2·31 ♂	2·63 ♂	2·28 ♀	—	6·92 ♀	—	*l-* 4·16 ♂	5·17 ♂	7·11 ♀
5	0·950	0·347	3	1·88 ♀	—	0·58 ♀	*dl-* 3·42 ♂	4·67 ♂	4·88 ♀	10·16 ♀	—	4·60 ♀	*dl-* 3·65 ♂	3·07 ♂	6·69 ♀
						2·13 ♂	*l-* 1·41 ♀	—	4·20 ♀	—	—	3·49 ♂	*l-* 4·51 ♀	—	4·34 ♂
6	0·948	0·350	3	1·06 ♀	—	2·89 ♂	*dl-* 2·16 ♂	1·35 ♀	2·98 ♀	7·76 ♀	—	3·12 ♂	*dl-* 4·35 ♂	4·64 ♀	5·42 ♀
						2·23 ♀	*l-* 2·38 ♂	—	5·04 ♀	—	—	5·88 ♀	*l-* 3·79 ♂	—	5·32 ♀
7†	0·805	0·339	3	0·76 ♀	0·22 ♀	3·05 ♀	*dl-* 3·44 ♂	2·38 ♀	3·44 ♀	7·24 ♀	5·27 ♀	4·43 ♀	*dl-* 4·81 ♂	1·87 ♀	3·80 ♀
						2·20 ♂	*l-* 1·63 ♀	4·06 ♀	4·31 ♀	—	—	2·70 ♂	*l-* 4·99 ♀	4·61 ♀	5·10 ♀
Mean	0·910	0·338	3	1·08	1·26	2·18	2·18	2·27	3·50	8·15	5·71	4·04	4·46	4·41	5·59

* By CCl_4 extraction and titration.

† Albinos; all the other litters were black and white.

Table II.

(Protocols.)

No. of exp.	Diet	Sex	Original weight g.	% loss of weight	% food eaten	% glycogen	% fat
1	Control	♀	90	3	100	1·31	
	Control	♀	78	2	100	1·30	—
	Acetate	♀	78	6	84	0·89	—
	Acetate	♀	94	7	72	1·54	—
	Acetate	♀	95	6	66	1·74	—
2	Control	♀	120	4	100	1·18	5·27*
	Control	♂	139	6	100	0·95	7·43
	Control	♀	105	3	100	0·22	11·05
	Acetate	♂	157	6	100	2·55	4·47
	Succinate	♂	149	9	94	2·46	4·07
3	Acetate	♀	102	9	98	0·74	6·89
	Acetate	♀	103	4	92	1·09	5·89
	Succinate	♀	105	10	82	1·49	4·95
	Succinate	♂	104	16	88	1·45	3·31
	l-Malate	♀	120	5	99	1·72	5·16
	l-Malate	♂	110	13	82	2·31	3·50
	Sugar control	♀	97	6	63	2·11	4·15
					(rat pneumonia)		
	Sugar control	♀	98	5	98	0·65	4·68
4	Acetate	♂	118	8	86	2·00	4·81
	Acetate	♀	119	7	90	0·56	6·92
	Succinate	♀	130	13	60	0·10	7·98
	Succinate	♂	130	12	44	2·63	5·17
	l-Malate	♀	125	7	90	1·05	5·69
	l-Malate	♂	138	9	78	2·31	4·16
	Sugar control	♀	120	4	100	1·63	9·29
	Sugar control	♀	121	0	100	2·28	7·11
5	Control	♀	70	2	98	1·88	10·16
	Succinate	♂	83	9	87	4·67	3·07
	dl-Malate	♂	80	9	66	3·42	3·65
	l-Malate	♀	75	9	78	1·41	4·51
	Fumarate	♀	82	12	57	0·58	4·60
	Fumarate	♂	76	9	69	2·13	3·49
	Sugar control	♀	72	1	99	4·88	6·69
	Sugar control	♂	70	0	91	4·20	4·34
6	Control	♀	105	7	100	1·06	7·76
	Succinate	♀	120	9	68	1·35	4·64
	dl-Malate	♂	140	12	49	2·16	4·35
	l-Malate	♂	138	11	79	2·38	3·79
	Fumarate	♂	115	10	49	2·89	3·12
	Fumarate	♀	117	10	79	2·23	5·88
	Sugar control	♀	115	2	100	2·98	5·42
	Sugar control	♀	110	0	100	5·04	5·32
7†	Control	♀	108	2	100	0·76	7·24
	Acetate	♀	112	4	84	0·22	5·27
	Succinate	♀	101	4	94	2·38	1·87
	Succinate	♀	108	5	72	4·06	4·61
	dl-Malate	♂	125	6	84	3·44	4·81
	l-Malate	♀	123	5	92	1·63	4·99
	Fumarate	♀	113	4	80	3·05	4·43
	Fumarate	♂	120	6	68	2·20	2·70
	Sugar control	♀	108	Gain of 2	100	3·44	3·80
	Sugar control	♀	103	Gain of 2	92	4·31	5·10

* In this group fats were estimated by extraction with carbon tetrachloride and titration with sodium hydroxide.

† Albinos.

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CLX. THE PHOSPHATIDES OF FORAGE GRASSES.
I. COCKSFOOT.

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A recent investigation by Channon and Chibnall [1927] of the phosphatides of cabbage leaves has shown that lecithin and kephalin are absent, and that the main constituent is a new phosphatide, calcium phosphatidate. In the preliminary investigations of the grasses the methods of these workers for the preparation and purification of leaf phosphatides were applied, but were not very successful for two reasons. In the first place the grass blades contained a higher proportion of solids, and especially of fibre, than cabbage leaves, consequently the grinding operations necessary to prepare the ether extract from the fresh material were very laborious; further, the percentage of phosphatide in the ether extract was low, so that 20 kg. samples of fresh grass gave only 2–4 g. of crude phosphatide. In the second place the treatment necessary for the preparation of pure phosphatidic acid resulted in the formation of emulsions. These are to be expected when working with any extract containing phosphatides, but in the present case they were far more difficult to break than those which had been encountered during the purification of the cabbage phosphatides. The presence of phosphatidic acid was readily proved, but the yields were disappointingly low, and attempts to separate and purify it by taking advantage of the water-solubility of the sodium salt showed that a large proportion of the crude phosphatide fraction was non-acidic in character, and was therefore essentially different from that found in the cabbage.

This result was to a certain extent unexpected, because in these early stages of the research we were working with products prepared from perennial ryegrass (*Lolium perenne*) and the analysis of the crude material was similar to that obtained from cabbage. (Found: C, 54·5; H, 8·3; ash, 19·8; Ca, 5·4; P, 3·9 %. A typical cabbage preparation gave C, 57·2; H, 8·7; ash, 19·5; Ca, 5·9; P, 4·6 %.) Later batches of material, however, from this and other grasses, often gave lower figures for ash, calcium and phosphorus, and in addition significant amounts of magnesium and nitrogen, *e.g.* batch *R* (Cocksfoot) gave C, 52·8; H, 8·1; ash, 12·5; P, 2·4; Ca, 1·0; Mg, 1·4; N, 1·0 %. These results, coupled with the observation that obstinate emulsions were

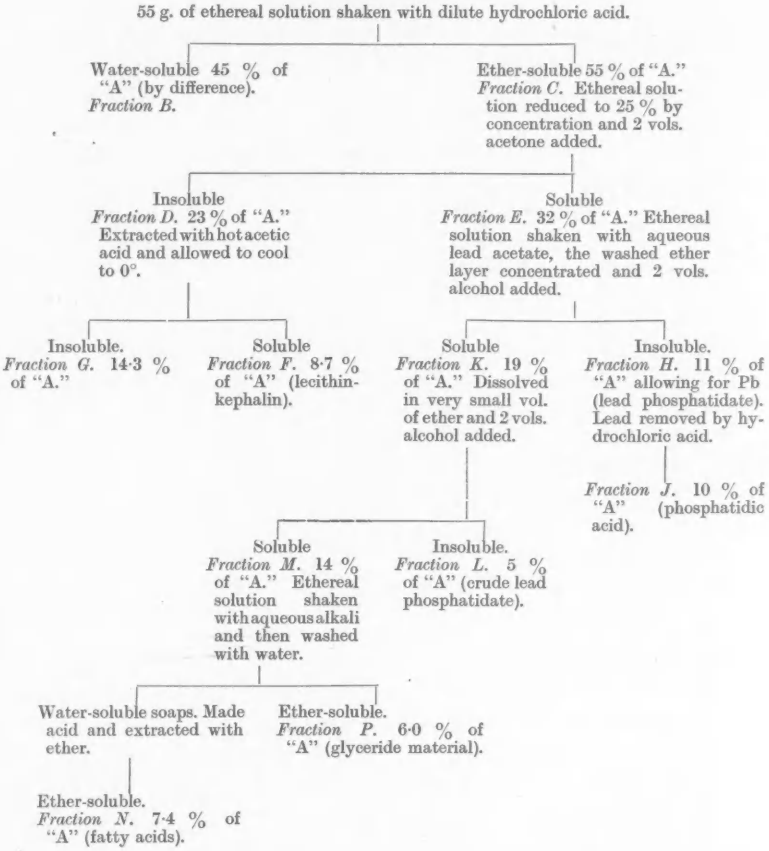
always formed when an ethereal solution of crude phosphatide was shaken with hydrochloric acid, led us to suspect the presence of lecithin and kephalin. To separate these two phosphatides from phosphatidic acid successfully demanded a larger quantity of crude phosphatide than we had hitherto been able to prepare by the methods we were then employing, and a change of procedure was necessary.

Now in connection with the general research which is being made in this laboratory into the composition of the ether extract of grasses we had already made numerous experiments to find out the best method of preparation; whether, for instance, the direct extraction of dried grass was preferable to the procedure of Chibnall and Channon [1927]. These results will be reported more fully in another communication, but at the above-mentioned stage in the phosphatide research it had been found that good yields of crude phosphatide could be obtained from grasses that had been dried very rapidly (1–2 hours) in a steam-oven through which a slow current of air was drawn, whereas the yields from grass dried slowly at room temperature or at 40° were poor. But the rapid drying of, say, 150 kg. of grass under these conditions was beyond the equipment of the laboratory, and we could not make use of dried grass as our raw material until we were fortunate enough to have placed at our disposal the new experimental grass-drying plant of the Imperial Chemical Industries at Jealott's Hill, in which the grass, supported on a moving belt, is passed along a chamber through which air heated initially to about 200° is blown. Drying is effected in about 8 minutes without "scorching" of the grass or any apparent chlorophyll breakdown. We were then able to obtain 22 kg. of dried cocksfoot (*Dactylis glomerata*), and from this 56 g. of crude phosphatide (Batch *R*). Knowing the extreme lability of phosphatides, as evidenced by the history of the "cuorin" and "jecorin" of the earlier workers in this field, we were apprehensive as to the fate of the grass phosphatides under this admittedly drastic drying treatment, and the procedure was adopted more or less under duress because it seemed at the time to be the only one available.

The large batch of crude phosphatide was readily fractionated by the method outlined in Table I, and we had no difficulty in showing that fraction F consisted chiefly of lecithin and kephalin by isolating derivatives of choline and β-aminoethyl alcohol after acid hydrolysis. The elementary analysis of this fraction showed that whereas the ratio N : P was practically 1 : 1 the actual amounts of N and P present were lower than were to be expected if it consisted only of these two phosphatides. This was found to be due, at any rate in part, to the presence of sugars or substances giving sugars on acid hydrolysis, which, calculated as glucose, were equivalent to 6·1 % of the fraction. This apparent adsorption of sugar by the phosphatide material will be referred to again later.

Having removed the major part of the lecithin and kephalin, and also fraction G, which will be shown later to consist of decomposition products, it

Table I. *Fractionation of crude phosphatide (fraction A).*


55 g. of ethereal solution shaken with dilute hydrochloric acid.
Water-soluble 45 % of "A" (by difference). Fraction B.
Ether-soluble 55 % of "A." Fraction C. Ethereal solution reduced to 25 % by concentration and 2 vols. acetone added.
Insoluble Fraction D. 23 % of "A." Extracted with hot acetic acid and allowed to cool to 0°.
Soluble Fraction E. 32 % of "A." Ethereal solution shaken with aqueous lead acetate, the washed ether layer concentrated and 2 vols. alcohol added.
Insoluble. Fraction G. 14·3 % of "A."
Soluble Fraction F. 8·7 % of "A" (lecithin-kephalin).
Soluble Fraction K. 19 % of "A." Dissolved in very small vol. of ether and 2 vols. alcohol added.
Insoluble. Fraction H. 11 % of "A" allowing for Pb (lead phosphatidate). Lead removed by hydrochloric acid.
Fraction J. 10 % of "A" (phosphatidic acid).
Soluble Fraction M. 14 % of "A." Ethereal solution shaken with aqueous alkali and then washed with water.
Insoluble. Fraction L. 5 % of "A" (crude lead phosphatidate).
Water-soluble soaps. Made acid and extracted with ether.
Ether-soluble. Fraction P. 6·0 % of "A" (glyceride material).
Ether-soluble. Fraction N. 7·4 % of "A" (fatty acids).

was possible to isolate phosphatidic acid by precipitation as the lead salt in the usual way (fractions H and J). We were thus able to obtain definite proof that the three phosphatides lecithin, kephalin and phosphatidic acid (as Ca or Mg salt) were all present in the blades of cocksfoot, which must, therefore, differ materially in their phosphatide metabolism from cabbage leaves, which contained only calcium phosphatidate.

While this result was satisfactory we were disappointed to find that the lecithin and kephalin (fraction F) and phosphatidic acid (fractions H and L) accounted for only about 9 % and 15 % respectively of the crude phosphatide (fraction A). An amount equal to 45 % passed into the aqueous phase on shaking with acid and the remainder into the three fractions G, N and P

which we believe to be products of decomposition brought about by the heating necessary to dry the grass and the subsequent treatment with aqueous acid. This conclusion is based partly on the exhaustive researches of Thudichum, MacLean and Levene, who, given adequate quantities of material, have been able to show that crude animal phosphatides contain only lecithin, kephalin and sphingomyelin, and that numerous other so-called individual phosphatides consist of lecithin and kephalin mixed with decomposition products which have been produced by the treatment necessary to prepare and purify the phosphatide material. Were it not for the fact that calcium phosphatidate was found by Channon and Chibnall to be the only phosphatide present in cabbage leaves, and that we have evidence, to be published shortly, that the lecithin and kephalin present in runner bean seeds give place first to magnesium and later to calcium phosphatidate during germination, we should have hestitated to suggest that fractions H and L represented phosphatidic acid and were not themselves products of kephalin decomposition.

Fraction G was a light grey powder insoluble in all organic solvents other than chloroform, and except that it had a N : P ratio nearly 1 : 1 instead of 1 : 2 resembled in other respects the "cuorin" isolated by Levene and Rolf [1926] from soya bean seed crude phosphatide. After acid hydrolysis it gave 8·1 % of reducing material calculated as glucose and only 20 % of fatty acids soluble in light petroleum. All the nitrogen was in the amino-form, and the fraction appeared to consist of kephalin fragments mixed with other products which had been rendered ether-soluble by the presence of true phosphatides in the original ether extract. Fraction P consisted of glycerides and fraction N of fatty acids. Both these fractions were freely soluble in ether-acetone, and could not have existed as such in the original ether extract; otherwise it is difficult to understand how they could have been precipitated by acetone with the waxes and phosphatides, and in any case have escaped extraction with the waxes during the hot acetone treatment. It seems reasonable to assume that they had been split off by the hydrochloric acid from phosphatide fragments which still retained the acetone-insolubility of the true phosphatides themselves. A study of the component fatty acids (as shown in Table II) lends support to this contention, for the acids from fractions N and P resemble those of the phosphatides F and J more closely than they do those derived from the glycerides of the true fat.

The mixed fatty acids from each of the four fractions F, J, N and P were oxidised under controlled conditions. Tetrahydroxystearic (sativic) acid and hexahydroxystearic (linusic) acid were readily isolated, showing that the original acids contained linoleic and linolenic acids. In a previous paper [Smith and Chibnall, 1932] we discussed at some length our inability to obtain any crystalline dihydroxystearic acid from the oxidation products of the true glyceride fatty acids of cocksfoot and perennial ryegrass and came to the tentative conclusion that if an octadecadienoic acid were present in the glycerides it was not oleic acid. Our experience in the oxidation of the phos-

Table II. *Oxidation of the fatty acids from various fractions of the cocksfoot phosphatides.*

Fraction	F (Lecithin-kephalin) %	J (Phospha-tidic acid) %	N (Free fatty acids) %	P (Glyceride material) %	Fatty acids from glycerides of true fat [Smith and Chibnall, 1932] %	Fatty acids from linseed oil %
Saturated acids	19	33	28	34	14	14
Dihydroxystearic acid fraction	3·3 (gum)	2·8 (gum)	5·4 (gum)	4·6 (gum)	3·6 (gum)	12·7 (crystalline M.P. 131°)
Tetrahydroxystearic acid	12·1	17·2	10·0	7·0	4·1	5·4
Hexahydroxystearic acid	4·3	6·7	9·0	10·0	3·4	5·9
Wt. of acids taken for oxidation (g.)	1·16	1·8	2·59	1·48	11·0	2·05
I.V. of acids taken for oxidation	144	128	137	125	185	176

phatide fatty acids again leads to the same conclusion. In each case the dihydroxystearic acid fraction consisted of a small amount of gum from which no crystalline material other than minute amounts of tetrahydroxystearic acid could be isolated. To obtain definite evidence that our technique was not at fault we oxidised a similar quantity of fatty acids derived from linseed oil, and isolated dihydroxystearic acid, M.P. 131°, without any difficulty. We therefore conclude that oleic acid is not present in the cocksfoot phosphatide fatty acids. It is interesting to note that Levene and Rolf [1925; 1926] in their analyses of the constituent fatty acids of soya bean lecithin and "cuorin" were unable to identify oleic acid.

The low proportion of saturated acids in the mixed fatty acids from cocksfoot phosphatides is in keeping with the results of Levene and Rolf for soya bean and of Channon and Chibnall for cabbage leaves, and emphasises the fact that plant lecithin and kephalin differ from those derived from animal sources in that they do not contain saturated and unsaturated acids in equimolar proportions.

The persistence with which most of these phosphatide fractions retain reducing sugars recalls the similar observations of Schulze and his co-workers, who worked with crude phosphatide preparations from seeds. Levene and Rolf [1925; 1926] were able to show, however, that in the case of the soya bean phosphatides this sugar was an impurity which could be removed by drastic purification, showing that no cerebroside or sugar-containing phosphatide was present. Sufficient evidence has been collected to suggest that this is also the case with the grass phosphatides.

When the crude phosphatide (fraction A) was shaken in ethereal solution with hydrochloric acid, material equivalent to 45 %, including reducing sugars equivalent to 8·1 %, passed into the aqueous phase. Fraction G on acid

hydrolysis gave 11 % and fraction F 6 % of reducing sugars, equivalent to 1·6 % and 0·5 % of fraction A respectively. Fraction A therefore contained at least 10 % of reducing sugars. The values for the sugar content of other batches of cocksfoot examined during the course of this work are collected in Table III. It will be observed that in the two cases in which the crude

Table III. *Sugar content of certain cocksfoot phosphatide fractions.*

Batch number	Treatment of grass	Solvent used for extraction	Reducing sugars in crude phosphatide (calculated as glucose) %
Q 1930	Fresh method of Chibnall and Channon [1927]	Ether	0·9
	Rapidly dried	Ether	3·1
A 1931	Fresh method of Chibnall and Channon [1927]	Ether	0·7
	Rapidly dried	Ether	3·0
	Rapidly dried	Light petroleum B.P. 40–60°	3·7

phosphatide was prepared by the procedure of Channon and Chibnall the sugar content was low, and when it is remembered that this method removes most of the water-soluble products, and therefore most of the leaf sugars, before the "coagulum" and residual leaf material are extracted with ether to prepare the crude phosphatide, it seems legitimate to assume that in the case of the dried grasses the sugar has been taken into ethereal solution through the agency of the lecithin and kephalin, which are known to possess unique solubility properties.

To summarise the results of our investigations on the crude phosphatide of rapidly dried grass, we have been able to show that it contains lecithin, kephalin and a salt of phosphatidic acid. Considerable amounts of decomposition products, probably derived from kephalin are also present, and for this reason it does not seem to us profitable to discuss either the relationship which the amount of crude phosphatide isolated bears to the amount of true phosphatide originally present in the fresh grass, or whether the phosphatidic acid was present as calcium or magnesium salt.

Experimental.

Before describing the fractionation of the phosphatides prepared from cocksfoot it is necessary to discuss briefly the theoretical analytical figures which are to be assumed for the lecithin, kephalin and phosphatidic acid present.

Investigation of the constituent fatty acids from each of the products isolated has shown that the I.V. was 125–145 and that approximately one-third was composed of saturated fatty acids M.P. 53–55°, the analytical figures for which in every case fall between those of palmitic acid $C_{16}H_{32}O_2$ (C, 74·9;

H, 12·6 %; mol. wt. 256), and stearic acid $C_{18}H_{36}O_2$ (C, 76·0; H, 12·8 %; mol. wt. 284). The saturated acids from the glycerides of the true fat [Smith and Chibnall, 1932] were similar and were shown to consist of about 70 % palmitic, 20 % stearic and 10 % higher acids. If therefore 25 % of the total acids from the three phosphatides be taken as palmitic acid, then it is probable that the remaining 75 % consists of C_{18} acids whose mean unsaturation is equivalent to three double bonds in two molecules, and it is on this basis that the following analytical figures have been calculated.

	C %	H %	N %	P %
Lecithin	65·3	10·9	1·77	3·91
Kephalin	65·6	10·6	1·91	4·24
Phosphatidic acid	66·5	10·4	—	4·52
Lead phosphatidate	51·2	7·7	—	3·48

Lead phosphatidate having this composition should give an ash of lead pyrophosphate equal to 33 %.

During the present research more than one hundred micro-combustions have been carried out on products obtained at various stages in the fractionation, and we have found it unusually difficult to obtain reproducible figures for carbon, hydrogen and ash. In almost all cases the carbon figure was liable to vary between 1 and 2 units in 50 or 60, while the ash showed a similar but not necessarily corresponding difference.

The following are quoted as typical examples:

		C %	H %	Ash %
Crude lead phosphatidate (Batch *P*)	1	50·3	7·8	30·5
	2	48·2	7·1	29·5
The same, after purification by precipitation from ethereal solution by alcohol	1	47·9	7·6	32·9
	2	47·0	7·6	32·6
	3	48·3	7·4	32·6
Theory requires		51·2	7·7	33·0

Throughout the investigation reported below we have not been able to handle sufficient material to enable us to prepare chemically pure lecithin, kephalin or phosphatidic acid. We have, therefore, been dealing throughout with mixtures, and the errors may be due, in large part, to the difficulty of obtaining a representative sample of such gummy substances in the small amount required for a micro-combustion. Loss of ash may also be caused by the creeping of the molten material from the boat before it is completely burnt.

Fractionation of the crude phosphatide from dried cocksfoot.

22 kg. of the dried cocksfoot (*Dactylis glomerata*) were exhaustively extracted with ether by British Drug Houses, Ltd. and gave 770 g. of ether extract. This was dissolved in 2 litres of warm ether and 4 litres of hot acetone were added with stirring. After standing overnight at 0° the precipitate of

wax and phosphatide was filtered off, redissolved in a similar quantity of warm ether and again precipitated with acetone. The resulting mixture was then extracted six times with hot acetone to dissolve out the wax fraction, each acetone extract being filtered hot. As the crude phosphatide was a sticky gum which might well retain some of the wax, it was thought advisable, after the second and fourth extractions with hot acetone, to dissolve the residue in ether and remove the solvent by evaporation, thereby exposing a new surface of the phosphatide to the action of the hot acetone. No appreciable amount of phosphatide passed into the hot acetone, although, as will be shown later, it contained both lecithin and kephalin. The crude phosphatide thus obtained (fraction A, Table I) weighed 56 g., equivalent to 7·3 % of the ether extract. It analysed as follows: C, 52·8; H, 8·1; P, 2·44; Ca, 1·02; Mg, 1·36; N, 1·0; ash, 12·5 %. The subsequent treatment of the material is outlined in Table I.

Treatment of the crude phosphatide with dilute hydrochloric acid.

55 g. of "A" were dissolved in ether, shaken four times successively with 500 cc. of dilute hydrochloric acid, and then well washed with water. Emulsification at this stage caused considerable trouble, but by varying the relative amounts of ether and of water present, by the judicious addition of small amounts of alcohol, and by the aid of the centrifuge, this difficulty was largely overcome. 55 % of the original material remained soluble in ether, so that by difference 45 % had passed into the acid and aqueous washings. These latter were collected and analysed. They contained 79 % of the calcium, 76 % of the magnesium, 41·5 % of the phosphorus and 26 % of the nitrogen originally present in the crude phosphatide. In addition, reducing substances, estimated as glucose by the method of MacLean [1919] were present to the extent of 8·1 % of the crude phosphatide.

The ethereal solution on evaporation gave 30 g. of material which analysed as follows: C, 63·0; H, 9·3; P, 2·73; Ca, 0·28; Mg, 0·12; N, 0·63; ash 3·9 %. This was dissolved in the minimum amount of ether and several volumes of acetone added. After standing overnight at 0° the precipitate was centrifuged and washed with acetone. The ether-acetone solution and the acetone washings were collected, the solvents removed, the resulting material again dissolved in the minimum amount of ether and precipitated with acetone. The material insoluble in ether-acetone constitutes the impure lecithin-kephalin fraction D, and the soluble material the crude phosphatidic acid mixture E.

Fraction D. This fraction represented 23 % of fraction A, and analysed as follows: C, 53·4; H, 8·8; ash, 9·3; P, 2·82; N, 1·5 %. It was extracted three times with boiling acetic acid, the extracts united, reduced to a small volume and allowed to stand at 0° for some hours. The material soluble in the cold acetic acid represents the lecithin-kephalin fraction F, and the material insoluble in both hot and cold acetic acid constitutes fraction G, which will be shown later to consist of decomposition products.

Lecithin-kephalin (*fraction F*). This fraction, representing 8·7 % of the original fraction A, was a black sticky gum which analysed as follows: C, 63·5; H, 10·1; P, 3·0; N, 1·4; ash, 6·0 %; N : P ratio 1·03 : 1. It was shown to consist chiefly of lecithin and kephalin by identification of the products obtained on acid hydrolysis.

The material (2·3 g.) was hydrolysed by boiling with 5 % sulphuric acid for 8 hours. The cooled mixture was then exhaustively extracted with ether, which removed 1·59 g. of fatty acids. The aqueous solution and washings were reduced *in vacuo* to 100 cc. and analysed for nitrogen and reducing sugars. The total N was 25 mg., the amino-N was 8 mg. and the non-amino-N (by difference) was 17 mg. Reducing substances, estimated as glucose by the method of MacLean [1919] were 140 mg. or 6·1 %. Glucose, and especially fructose, when submitted to the hydrolysis mentioned above would be partially destroyed, so that the low values for phosphorus and nitrogen in this lecithin-kephalin preparation are due, at any rate in large part, to the presence of carbohydrate.

Identification of β-aminoethyl alcohol. A sample of synthetic β-aminoethyl alcohol, kindly provided by Dr Dudley, was found to be quantitatively precipitated by the mercury carbamate method employed by Neuberg and Kerb [1912] for amino-acids, and the aqueous hydrolysis products of fraction F were accordingly treated in the following way.

Sulphuric acid was removed from solution with baryta, the barium sulphate being centrifuged and well washed with hot water. Lead acetate was then added, the small precipitate centrifuged, and lead removed from the clear liquid by means of hydrogen sulphide. The water-clear solution thus obtained was concentrated to 100 cc. and treated alternately with small amounts of 20 % aqueous sodium carbonate and 20 % aqueous mercuric acetate. The reaction was kept just alkaline to litmus and the reagents added until the addition of either produced a permanent orange precipitate. An equal volume of alcohol was then added and the mixture allowed to stand overnight. The precipitate was centrifuged off, washed with a little 50 % alcohol, suspended in water and mercury removed with hydrogen sulphide. The mercuric sulphide was well washed with hot water, the clear liquid and washings acidified with a little hydrochloric acid and evaporated to dryness *in vacuo*. The hydrochloride of β-aminoethyl alcohol was then extracted from the residue of inorganic salts with alcohol. The alcoholic extract was run into a small beaker, evaporated to dryness on the water-bath, the residue taken up in a few cc. of dilute hydrochloric acid and gold chloride added. The mixture was allowed to evaporate slowly in a vacuum desiccator. Brown cubic crystals came out during the course of a few days, and long orange needles were obtained on recrystallisation from water. The melting-point alone, and when mixed with authentic β-aminoethyl alcohol chloroaurate was 189–191° with previous softening at 187°. (Found: Au, 48·7. $C_2H_7ON.HAuCl_4$ requires Au, 49·2 %.)

Identification of choline. The aqueous-alcoholic filtrate from the mercuric

acetate precipitation mentioned above was concentrated *in vacuo* to 20 cc. and treated with a hot concentrated alcoholic solution of mercuric chloride. A white crystalline precipitate appeared almost at once, and increased on cooling. The mother-liquor on concentration gave another crop of crystals, and the total yield was 1·3 g. The crystals were typical of choline mercuric chloride and melted at 252° with previous softening. For confirmation 0·5 g. of the material was dissolved in a few cc. of hot water, mercury removed with hydrogen sulphide and the calculated amount of gold chloride added to the clarified liquid. Fine yellow needles of choline chloroaurate appeared on standing, and were filtered off. The mother-liquor on concentration gave a further yield of beautiful yellow plates. Both needles and plates melted at 269–270° with decomposition. (Found: C, 13·9; H, 3·2; Au, 44·6. $C_5H_{14}ON.AuCl_4$ requires C, 13·5; H, 3·2; Au, 44·5 %.) Allowing for aliquots withdrawn for analysis the yield of choline mercuric chloride represented 70 % of the non-amino-N present in the hydrolysis products of fraction F.

Identification of the fatty acids. The 1·59 g. of fatty acids mentioned above (I.V. 144; mol. wt. 290) represented a yield of 69 %. The theoretical yields for lecithin and kephalin are 69 % and 75 % respectively. For further identification of the constituent acids of this and other mixed fatty acids derived from the crude phosphatide the acids were oxidised with dilute alkaline permanganate below 10° as described by Lapworth and Mottram [1925]. The oxidation was allowed to proceed for 5 minutes when it was stopped by decolorising with sodium bisulphite followed by a little concentrated hydrochloric acid. The oxidation products insoluble in water were filtered off, dried in a vacuum desiccator, and the saturated acids extracted with light petroleum (B.P. 80–100°). These were then subjected to the magnesium soap treatment of Bertram [1927], to give an approximate value for the amount of saturated acid present in the original mixed fatty acids. The residue insoluble in light petroleum was next extracted three times with boiling ether to remove any dihydroxystearic acid. The material thus obtained in this and other similar fractions from the crude phosphatide was a brown gum, and attempts to prepare crystalline material resulted in the isolation of minute amounts of tetrahydroxystearic acid, whereas in the case of linseed oil, crystalline dihydroxystearic acid, M.P. 131°, was obtained without difficulty. The material insoluble in ether was recrystallised from alcohol and in each case was found to consist of mixed α- and β-sativic acids. The oxidation products which remained soluble in water were obtained by evaporating the solution to dryness *in vacuo* and extraction with alcohol. In all cases either α- or β- or both linusic acids were obtained.

In the case of the mixed fatty acids from fraction F, 1·16 g. were oxidised and gave (1) 0·21 g. of saturated acids which was recrystallised from alcohol. (Found: C, 74·5; H, 12·6 %; mol. wt. 282; M.P. 53–55°.) (2) 0·04 g. of dark gum representing the dihydroxy-derivatives. (3) 0·09 g. of tetrahydroxystearic acid M.P. 155–165°. (Found: C, 62·2; H, 10·7. $C_{18}H_{36}O_6$ requires C,

62·0; H, 10·4 %.) Fractionation from ethyl acetate gave α- and β-sativic acids of M.P. 157–159° and 172–173° respectively. (4) 0·05 g. of hexahydroxystearic acid. M.P. 185–190°. By repeated recrystallisation from water α-linusic acid, M.P. 203–204°, was obtained. (Found: C, 56·9; H, 9·7. $C_{18}H_{36}O_8$ requires C, 56·8; H, 9·5 %.)

Phosphatidic acid (fraction J).

The crude phosphatidic acid (fraction E) mentioned above represented 32 % of the crude phosphatide (fraction A). The analysis, C, 68·5; H, 10·3; P, 2·02; ash, 2·6 %, showed that some other fatty material, possibly glyceride, must have been present. This mixture, dissolved in ether, was shaken four times with a strong solution of lead acetate. The ethereal solution was well washed with water, concentrated to a small bulk and three volumes of alcohol added. The precipitate of lead phosphatidate was centrifuged, redissolved in a similar volume of ether and again precipitated with alcohol. The weight of lead salt (fraction H) so obtained, allowing for the lead present, represented 11 % of the crude phosphatide (fraction A). (Found: C, 50·7; H, 7·5; ash, 31·4. Lead phosphatidate requires C, 51·2; H, 7·7; ash, 33·0 %.) An ethereal solution of the salt was shaken with dilute hydrochloric acid to remove the lead, washed with water, and evaporated to dryness *in vacuo*. The amount of phosphatidic acid (fraction J) was equivalent to 10 % of the crude phosphatide (fraction A). (Found: C, 63·9; H, 9·8; ash, 6·6; P, 4·24; N, 0·6. Phosphatidic acid requires C, 66·5; H, 10·4; P, 4·52, N, 0 %; mol. wt. 686.) The I.V. was 98 and the mol. wt. by titration 746, showing that the preparation still contained some non-acidic material. This was confirmed when 3·46 g. were saponified with strong alcoholic potassium hydroxide for 2½ hours. 1·91 g. of mixed fatty acid, representing only 77 % of the theoretical yield, were obtained. The I.V. was 128 and the mol. wt. 298.

Identification of the fatty acids. 1·80 g. of mixed fatty acids were oxidised by the methods outlined above, and gave (1) 0·60 g. of saturated acids, M.P. 52·5–54·5°. (Found: C, 75·6; H, 13·0 %.) (2) a gum weighing 0·06 g. representing the dihydroxystearic acid fraction. (3) 0·31 g. of mixed α- and β-sativic acids, which were separated by means of ethyl acetate. (4) 0·12 g. of hexahydroxystearic acid. Several recrystallisations from water and alcohol gave products melting at 197° and 167° respectively. (Found, for the latter: C, 56·8; H, 9·8 %.) The melting-points of α- and β-linusic acids are stated to be 203° and 173° respectively and in view of the small amount of material available it is probable that the fractionation had not been carried far enough.

Phosphatide decomposition products.

Under this heading are described the three fractions which appear to be products of phosphatide decomposition brought about by heat during the drying of the cocksfoot.

Fraction G. This fraction was insoluble in glacial acetic acid, and represented 14 % of the original crude phosphatide (fraction A). It was a light grey powder which absorbed chloroform to form a gel but which did not readily disperse into solution. Otherwise it was insoluble in the usual organic solvents. (Found: C, 51·0; H, 8·3; ash, 3·5; P, 2·15; N, 1·1 %.) The product resembled in many respects the so-called "cuorin" fraction which Levene and Rolf [1926] isolated from soya bean seed phosphatides, which was soluble only in chloroform, had a ratio N : P of 1 : 2 and which they considered to be a mixture of kephalin decomposition products. In the present case the decomposition had been more extensive and the product was contaminated with substances giving reducing sugars on acid hydrolysis.

4·4 g. of material were hydrolysed by boiling with 10 % hydrochloric acid for 8 hours, the mixture cooled and extracted with ether. 0·25 g. of a black powder remained insoluble in both ether and water, while the ether extracted 1·31 g. of material which was molten at 100° and semi-solid at room temperature. This latter gave 0·92 g. of fatty acids soluble in light petroleum, a yield of only 21 %, showing that the phosphatide fragments had lost part of their complement of fatty acids. The aqueous acid contained reducing substances equivalent to 0·48 g. of glucose and also 0·043 g. of nitrogen, all of which was in the amino-form.

Fraction N (free fatty acids). Fraction K, the mother-liquor from the precipitation of the lead phosphatidate (fraction H), was concentrated to a small volume and three volumes of alcohol added. A further quantity of crude lead phosphatidate (fraction L) was precipitated. (Found: C, 55·1; H, 8·3; ash, 23·5 %.) The clear alcoholic solution (fraction M) represented 14 % of the crude phosphatide (fraction A). (Found: C, 73·8; H, 11·1; ash, 1·2 %.) The analysis suggested that the product was a mixture of fatty acids or glycerides. 5·20 g. were accordingly dissolved in ether and shaken with aqueous sodium hydroxide. 2·25 g. of neutral material (fraction P) remained in the ether and 2·76 g. of fatty acids (fraction N) were recovered from the alkaline soap solution. 2·59 g. of these acids were oxidised and gave (1) 0·72 g. of saturated acids (Found: C, 75·4; H, 12·4 %; mol. wt., 284; M.P. 53·5–55·5°); (2) 0·14 g. of dark gum representing the dihydroxystearic acid fraction; (3) 0·21 g. of tetrahydroxystearic acid, M.P. 165° indefinite (Found: C, 61·8; H, 10·5. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %); fractionation from ethyl acetate and alcohol gave α- and β-sativic acids, of M.P. 158–160° and 173° respectively; (4) 0·27 g. of crude hexahydroxystearic acids; M.P. 184° with previous softening at 165°.

Fraction P (mixed glycerides). The neutral material from the above separation represented 6 % of fraction A. (Found: C, 74·9; H, 11·8 %.) On mild saponification 2·1 g. gave 1·63 g. of mixed fatty acids (I.V. 125; mol. wt. 280) and 0·25 g. of unsaponifiable material, probably a wax alcohol. (Found: C, 81·3; H, 12·8 %.) The saponification value was 167, or, allowing for the small amount of unsaponifiable material, 185. These results suggest that the

saponifiable material consisted chiefly of diglyceride fragments split off from the phosphatide. A monoglyceride requires C, 70·7; H, 11·3 %; sap. val. 156: a diglyceride C, 75·4; H, 11·7 %; sap. val. 180: and a triglyceride C, 77·1; H, 12·3 %; sap. val. 189.

Oxidation of 1·49 g. of the fatty acids gave (1) 0·51 g. of saturated acids (Found: C, 75·1; H, 12·8 %; M.P. 59–60°; mol. wt. 259); (2) 0·07 g. of gum representing the dihydroxystearic acid fraction; (3) 0·105 g. of mixed α- and β-sativic acids, M.P. 157–163°, (4) 0·145 g. of mixed α- and β-linusic acids, M.P. 173°. The composition of these glyceride fatty acids, and also of the free fatty acids of fraction N, more closely resembles that of the phosphatides than that of the glyceride components of the cocksfoot fat (compare Table II).

Oxidation of fatty acids from linseed oil. 2·35 g. of linseed oil gave on saponification 2·17 g. of mixed fatty acids of I.V. 176. 2·05 g. of these acids were oxidised and gave (1) 0·29 g. of saturated acids, M.P. 52–53°, (2) 0·26 g. of dihydroxystearic acid; recrystallised repeatedly from alcohol it melted at 130·5–131° (Found: C, 68·3; H, 11·4; $C_{18}H_{36}O_4$ requires C, 68·5; H, 11·5 %); (3) 0·11 g. of mixed α- and β-sativic acids, M.P. 168–170°; (4) 0·12 g. of mixed α- and β-linusic acids, M.P. 192°.

SUMMARY.

The phosphatide fraction prepared from a sample of rapidly dried cocksfoot has been investigated in some detail.

Considerable phosphatide decomposition appears to have occurred during the drying, but lecithin, kephalin and the Mg or Ca salt of phosphatidic acid have been definitely identified.

The constituent fatty acids of the phosphatides have been investigated by controlled oxidation. Saturated acids, linoleic acid and linolenic acid were present. As in the case of the glyceride fatty acids of cocksfoot, no evidence for the presence of oleic acid could be obtained.

The ratio of saturated to unsaturated acids was less than unity, as appears to be usual in the case of plant phosphatides.

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CLXI. THE GONADOTROPIC HORMONES (ρ-FACTORS).

III. FURTHER PURIFICATION AND PROPERTIES OF THE ACTIVE PRINCIPLES.

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Since the original observation by Zondek [1930] that the gonadotropic substances of pregnancy urine are precipitable by alcohol in sufficient concentration, numerous processes, based on this and other observations, have been devised by other workers with the object of obtaining a highly active substance in such a condition that it could be stored without loss of potency. Dickens [1930] made preparations by the alcohol precipitation process, and also by methods based on precipitation with ammonium sulphate or tannic acid. He obtained a product which, on many occasions, had a luteinising action in the immature mouse in doses of 0·015 mg., and in one instance only of 0·006 mg. Wiesner and Marshall [1931] by considerable modification of the alcohol precipitation process also obtained a product of comparable activity on many occasions, and in one instance by a process based on the precipitation of the active factors with phosphotungstic acid.

Fischer and Ertel [1931], in a comprehensive communication, describe various attempts at purification of commercial samples of the gonadotropic hormones which contained 4–5 m.u. per mg.; they obtained products containing, in some cases, as much as 130 m.u. per mg. by removal of phosphates with baryta or barium acetate, a method which was previously used by Wiesner and Marshall [1931], who, however, removed the barium more completely by the use of sulphuric acid. Some measure of purification was also obtained by Fischer and Ertel by adsorption of the active principles on kaolin or bolus alba at p_H 4·5 and elution with dilute ammonia. Electrodialysis at temperatures $< 10°$ of a product containing 120 m.u. per mg. failed to effect further purification and even caused a destruction of active substances.

Marshall [1931] has shown that solutions of ρ-factor preparations can be selectively filtered through a series of "membran" and "ultrafein" filters supplied by the Membranfilter Gesellschaft, the coarser grades passing the active factors completely and the finer only to a small extent, if at all. In

the course of this work it was observed that even in those cases where the ρ-factors were almost quantitatively retained by the filter, the filtrate was quite strongly coloured. This observation suggested the possibility of removing a large part of the coloured organic impurities in this way, such impurities being, incidentally, the cause of the original hygroscopic nature of the products. The filters which seemed likely to be of use in this connection were "ultrafein mittel," "ultrafein fein," and "ultrafein Benzopurpurindicht." The former two were actually used in the experiments to be described.

The possibility of further purification *via* the phosphotungstates was also investigated but the results obtained were far inferior to those from the filtration technique.

Attempts at purification by combination with permutit in acid solution, with subsequent extraction with dilute ammonia were entirely unsuccessful.

Animal standardisation.

In order to determine the potency of different preparations, various dilutions were made in 0·85 % saline and injected in convenient amounts into several series of 5 mice each. The total volume injected into each animal was 0·25–0·50 cc. and this was divided into 5 doses given over a period of 2½ days. With the highly active products the solution injected contained 0·01 mg. of solid substance per cc. After 2–3 days following the last injection the animals were killed, a positive result being regarded as one in which at least two animals of a series showed an ovarian response (*i.e.* corpora lutea or atretica). In some cases, as will be seen later, cornification of the vaginal epithelium was observed with a dose less than that required to produce an ovarian effect.

It should be borne in mind that accurate standardisation is out of the question where so many different factors are involved. This is particularly the case when one considers that, on present conceptions, there are two factors responsible for the effects upon the ovary, and that these factors are antagonistic to an extent which we are as yet unable to measure quantitatively. At the present time, although separation of ρ_1 and ρ_2 cannot be effected, we have no certainty that the relative proportions of the two are constant in different preparations. Although some such approximate standardisation is necessary in the absence of a more satisfactory method, its limitations must be recognised and an endeavour made to standardise at least the factors which can be controlled, such as the number of injections into which a dose is divided and which have been shown to exercise such a great effect in the "standardisation" of oestrin (α).

EXPERIMENTAL.

Experiments with permutit. A solution of 1·0 g. ρ-factors (20 units per mg.) in 10 cc. water was acidified to p_H 4·5 with sulphosalicylic acid, shaken vigorously at intervals with permutit and allowed to stand for half an hour. The permutit was removed by centrifugation, washed with water acidified with

sulphosalicylic acid, and extracted with 10 cc. of 1 % ammonia (sp. gr. 0·880, 1 : 100) by shaking. After centrifugation the supernatant fluid was neutralised with 10 % acetic acid and 4 volumes of absolute alcohol and 1 volume of ether were added. The precipitated product was washed with alcohol, then with ether, and dried. A dose of 0·025 mg. failed to produce any ovarian response in the immature mouse. No attempt was made to determine whether the product was active in larger doses, since the sole object of the experiment was to investigate the possibility of utilising the method as a basis for further purification.

Experiments with phosphotungstic acid. A solution of 1·0 g. ρ-factors (20 units per mg.) in 9 cc. of water and 1 cc. of 10 % sulphuric acid was centrifuged to remove a small amount of insoluble matter, and fractional precipitation resorted to. Fraction *a* was produced by addition of 2 cc. of 20 % phosphotungstic acid; fraction *b* by addition of 3 cc. of phosphotungstic acid to the supernatant fluid from *a*; fraction *c* by further addition of 5 cc. of the acid to the supernatant fluid from *b*. All three fractions were washed with absolute alcohol and dried. They were then suspended in water; solution was effected by cautious addition of 10 % ammonia (sp. gr. 0·880; 1 : 10), and phosphotungstic acid removed with barium hydroxide. The excess of barium was quantitatively removed with sulphuric acid and the active ρ-factors were precipitated by addition of 4–5 volumes of alcohol. Washing and dehydration were effected by means of alcohol and ether as previously described. Fractions *a* and *b*, tested in immature mice, were found to contain 40 units per mg. Fraction *c* was almost inactive.

Filtration experiments. The products used in the filtration experiments were all entirely free from protein and usually had a potency of about 20 units per mg.; they were prepared by the alcohol precipitation method, involving the use of barium hydroxide, previously described by Wiesner and Marshall [1931] for the preparation of their *E* 198. The following is a résumé of the technique which has been employed in some dozen instances. A solution of 2 g. of ρ-factors in 100 cc. of distilled water (adjusted to p_H 7·2) was filtered at the water-pump on two filters ("ultrafein mittel" or "fein"), the effective filtering area of which was about 7 cm. in diameter. When the volume of residual liquid on each filter was reduced to 5–10 cc., distilled water was added and the filtration allowed to proceed until the residual volume was not more than 5 cc. This washing process was repeated three times, by which time the filtrate was colourless. The residual liquid was thoroughly washed off the surface of the filters and the solution centrifuged until quite clear. Addition of 5 volumes of absolute alcohol gave merely an opalescent solution, but the purified ρ-factors were precipitated by the further addition of an equal volume of ether. The operation up to this point took about 7–8 hours. The precipitate was removed by centrifugation, washed with alcohol and ether, and dried. The yield was usually 0·15–0·2 g. The potency of the product, in every case in which filtration was as complete as described above, was 200 units

(luteinising) per mg. It is interesting to note that in two experiments 60 % of the animals receiving 0·0025 mg. of active substance showed complete cornification of the vaginal epithelium, the vaginae being well open in all cases 3 days after the cessation of injections. This means that the oestrogenic potency of these samples is 400 units per mg. and it also confirms Wiesner and Marshall's findings with regard to the differential effects of small and large doses with cruder preparations, although in this series of experiments, which deals with liminal doses as regards luteinising activity, the vaginae were open without exhibiting cornification in the larger doses. In the course of this work it has been noticed that the filtrates frequently possessed a certain degree of activity. This may be due to two causes, (1) the relatively concentrated solutions used for filtration (400 units per cc.) whereby activity in the filtrate was more readily observed in the doses given, and (2) the slightly increased permeability of the membranes after continued use. A product was obtained from these filtrates which was compared for its nitrogen content with the highly active substance remaining on the filter.

Chemical investigation of the products obtained.

Nitrogen content. Determinations of nitrogen were made on a considerable number of specimens using a micro-Kjeldahl method. Merck's perhydrol (0·5 cc.) was used to assist the oxidation as it was found to be very effective for this purpose, whilst containing only minute traces of nitrogen. Table I gives the values obtained with the original product, the product from the filtrate and the highly active residue on the filter.

Table I.

Substance	N content %	Activity (units per mg.)
Original product (102)	5·2 5·3	*ca.* 20
Filtrate (102 *b*)	4·6 4·6	>2
Filtrate (102 *e*)	4·4 4·4	<2
Active residue (102 *f*)	8·4	200
Active residue (102 *a*)	8·3 8·5	200
Active residue (102 *d*)	7·9 8·1	145

The figures given are consistent with the hypothesis that the active substance is a N-containing compound, the highest percentage being found in the most active product, and the lowest in the relatively inactive product from the filtrate. Specimen 102 *d* was submitted to less efficient washing in the process of filtration and has therefore a somewhat lower activity than 102 *a* and *f*. Many other specimens with an activity of 200 units per mg. gave similar results. No allowance has been made in the above figures for ash content of the specimens. No. 102 *f* had an ash content of 7·16 %, the residue

containing no calcium or barium and only a minute trace of iron; the N content on an ash-free basis is therefore 9·09 %. Fischer and Ertel [1931] give N values of 10·0–11·10 % on an ash-free basis for a product with an activity of 120 units per mg.

Qualitative tests (on the highly active product).

(1) No halogens, phosphorus, or sulphur are present.

(2) No pentoses (phloroglucinol and Bial's tests) are present.

(3) Millon's test is definitely positive.

(4) Molisch's test gives a colour which is much redder than that given by glucose.

(5) Phosphotungstic acid, in presence of sulphuric acid, gives a flocculent white precipitate which is biologically definitely active.

(6) The glyoxylic acid reaction is very doubtful.

(7) Addition of 5 volumes of alcohol alone to a relatively concentrated solution (2000 m.u. per cc.) does not precipitate the active factors. Further addition of ether or a small volume of saturated sodium chloride solution is required; in actual practice ether was always used.

(8) The ninhydrin reaction for α-amino-acids is negative alone, but following brief boiling with dilute sulphuric acid and subsequent neutralisation the reaction is obtained.

(9) Knoop's bromine test for histidine is negative.

Tyrosine and tryptophan content. An approximate tyrosine and tryptophan determination was made on the highly active product by Folin and Marenzi's method [1929]; results: tyrosine, 1·25 %; tryptophan, 0·6 %. Owing to the small amount of material which remained after the completion of other experiments, it was not possible to repeat these analyses with extraction of the alkaline hydrolysates with toluene, which procedure was found by Macfarlane and Fulmer [1930] to be necessary for obtaining accurate tryptophan values with certain proteins. The figure recorded above is, therefore, almost certainly too high, and the rather doubtful qualitative glyoxylic acid reaction obtained is explained.

Attempt to demonstrate the presence of carbohydrate. A partially purified specimen (20 units per mg.) was first used and the following technique adopted [*cf.* Osborne and Campbell, 1900]. 1 g. of the product (20,000 m.u.) was boiled under reflux with 20 cc. of 10 % sulphuric acid. After hydrolysis the sulphuric acid was removed quantitatively with barium hydroxide and the precipitate of barium sulphate and humin centrifuged off. The precipitate was washed once with 10 cc. of distilled water. The clear supernatant fluid (p_H 7·0–7·5) was evaporated to 10 cc. and 1·0 g. of sodium acetate and 0·5 g. of phenylhydrazine hydrochloride were added. The hot solution was immediately filtered from some tarry material and then further heated at 100° for 1 hour and allowed to cool overnight. The precipitate was collected and washed with water and a little 95 % alcohol. To the solution in boiling alcohol

1–2 parts of hot water were added and the solution was allowed to cool slowly. The yellow-brown product which separated had the typical crystalline form of glucosazone. After a further crystallisation the product had M.P. 183–185°; yield 10 mg.

A similar technique using 0·4 g. (80,000 m.u.) of the highly purified product failed to give any trace of osazone. Fischer and Ertel [1931] were also unable to obtain an osazone from a product with an activity of 100–120 m.u. per mg.

Attempt at crystallisation. 2 cc. of saturated ammonium sulphate were added to 0·1 g. of highly active ρ-factors in 1 cc. of water (p_H 7·0). After standing for 2 days at 0° some amorphous product had separated but no crystalline material could be detected.

Biological experiments.

In addition to the standardisation of preparations already referred to, a number of preparations were tested for their efficacy in inducing ovulation in the rabbit. With samples of which 1 m.u. = 0·005 mg. it was found that 0·1 mg. would produce rupture of one or more follicles with probable ovulation although ova were not isolated. It is proposed later to determine whether any relationship exists between the mouse unit (luteinising) and the rabbit unit (ovulation) with a view to obtaining evidence of the identity, or otherwise, of the factors. This point is of particular interest in view of Leonard's [1931] suggested differentiation.

Discussion.

The filtration technique described above has been found extremely useful in the preparation of small quantities of a highly active, almost salt-free product. The use of the ultrafiltration method also enabled the author to remove some coloured resinous impurities which could not be removed by ordinary dialysis through collodion or cellophane membranes. As a result of this work it has been made clear that the precipitability of ρ-factors from protein-free solutions by alcohol is dependent on at least a small inorganic salt content, since sodium chloride can replace ether as a precipitating agent for the opalescent solutions obtained by the addition of alcohol alone. The fact that the ninhydrin reaction was only obtained after a brief hydrolysis with mineral acid is important as support for the view that the product is polypeptide in nature. When, in previous work, a qualitatively very definite positive Millon's reaction was obtained, it seemed possible that tyrosine formed an intrinsic part of the active molecule, as was also suggested by Fischer and Ertel. The roughly quantitative determinations reported here would seem to cast some doubt on this hypothesis, although the presence of a polypeptide has been more or less definitely established. Further, that such polypeptide, if it may be assumed to be the active principle (or principles), does not possess its activity merely by virtue of the presence of the fundamental —CO—NH— linkages which it contains, is obvious from the finding that a solution of

purified ρ-factors loses its entire activity on heating at 100° for half an hour, whereas such heating does not decompose the polypeptide sufficiently to give the ninhydrin reaction. If, therefore, our assumption that the active principles are polypeptide in nature is correct, we must of necessity postulate some other rearrangement of the molecule or splitting off of some side-chain in a key position without effect on the polypeptide linkage. The only other alternative would be that the active principles of unknown nature were adsorbed on the polypeptide.

It seems hardly likely that any polysaccharide forms an essential part of the molecule, as suggested by Fischer and Ertel, in view of their own failure to obtain any osazone following either brief or prolonged hydrolysis, but also by reason of the author's demonstration of the presence of a sugar in a partially purified sample (containing 20,000 m.u.) coupled with his inability to do so in a highly purified sample containing 80,000 m.u.

Summary.

1. A further purification of ρ-factor preparations by an ultrafiltration method is described. "Ultrafein mittel" and "fein" filters of the Membranfilter Gesellschaft are used. Specimens containing 200 m.u. per mg. have been obtained.

2. Neither the use of phosphotungstic acid nor that of permutit yielded sufficiently satisfactory results.

3. The presence of a polypeptide in the highly purified product is established by the application of the ninhydrin reaction before and after hydrolysis.

4. The presence of a sugar complex in the active substance is doubtful.

5. Not more than 1·25 % of tyrosine is present in the purified product which also contains about 8·4 % of nitrogen and 7 % of ash. The presence of tryptophan is very doubtful.

6. 0·1 mg. of the product will induce rupture of the follicles with probable ovulation in the rabbit within 24 hours.

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CLXII. HISTAMINASE.

I. THE HISTAMINE-HISTAMINASE REACTION. II. PURIFICATION. III. SPECIES DISTRIBUTION.

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Histaminase is the name which has been applied [Best and McHenry, 1930] to a histamine-inactivating enzyme. Originally found by Best [1929] to be present in ox and horse lung, investigations on the histaminase content of dog tissues showed it to be most plentifully contained in the kidney and in the intestinal mucosa. These studies, utilising fresh tissue *in vitro*, led to perfusion experiments in which it was found that isolated dog kidneys could inactivate large amounts of histamine. The obvious suggestion has been made that this enzymic mechanism, functioning principally in the kidney and in the wall of the small intestine, may constitute one of the body's protections against harmful amounts of histamine.

Some of the properties of histaminase have been previously described. It is heat-labile, being irreversibly inactivated at 60°. For its activity the optimal temperature is 38°, and the optimal p_H range is from 6·9 to 8·0. The enzyme is inhibited slightly by ammonium and magnesium ions, and markedly by calcium, while it is not affected by sodium and potassium. Of the anions, chloride is without effect, sulphate and citrate are inhibitory, and phosphate definitely augments the reaction. As it was found that the histamine-histaminase reaction does not occur in presence of $M/2000$ potassium cyanide, proceeds very slowly in an atmosphere of nitrogen, and is accelerated by oxygen, it has been thought to be oxidative. Under certain conditions, if not always, the enzyme causes disruption of the iminazole ring in the histamine molecule.

Employing the Barcroft-Warburg technique, Gebauer-Fuelnegg and Alt [1932] have measured oxygen consumption during the histamine-histaminase reaction. The enzyme was prepared from dog kidney. No simple molar relation was demonstrated between the amount of oxygen consumed and the amount of histamine inactivated. Three possible discrepancies were noted by these authors between their conclusions, based on oxygen-uptake measurements during the histamine-histaminase reaction, and results obtained from other methods of investigation, which had been reported from this laboratory:

(1) in their experiments more histamine was inactivated at p_H 10 than at 7·4; (2) more histamine was inactivated at 50° than at 37°; (3) potassium cyanide did not completely inhibit the reaction. Since the publication of these results we have repeated the measurements of inactivation at varying temperatures and at varying hydrogen ion concentrations and have found no disagreement with our earlier results.

Several observations by Gebauer-Fuelnegg and Alt are of interest. The enzyme seems to be specific for histamine, as indicated by the lack of oxygen-uptake when active kidney-powder was incubated with several other iminazole derivatives. We had previously found that histaminase does not affect tyramine, an amine possessing the same side-chain as is found in histamine. The results of the brief study by Gebauer-Fuelnegg and Alt of species distribution of histaminase are not in agreement in the case of several species with the data presented in the present paper.

This communication gives results of further studies on the histamine-histaminase reaction, descriptions of some purification procedures and data on the variation in the histaminase contents of kidneys of several species of animals.

As a source of histaminase, a dry powder prepared from ox kidney by extraction of the fats with acetone, removal of acetone with ether and subsequent drying of the tissue was employed in earlier experiments. One powder prepared in this manner showed no loss of activity during storage for one year at laboratory temperature. To secure a stable powder it is essential that the acetone extraction be thorough and that the powder be rapidly dried to remove ether. More recent work has shown that hog kidney is a better source and histaminase powder is now prepared from that tissue by the method described above. The enzyme is removed from the powder by extraction at 38° with phosphate buffer of p_H 7·0 to 7·2. The amount of enzymic action is determined by measuring the amount of histamine remaining after incubation of a mixture containing a known amount of histamine and the tissue or enzyme preparation. All determinations of histamine have been made by the blood-pressure method in etherised cats. For the assays solutions were prepared from the mixtures after incubation by the hydrochloric acid procedure [Best and McHenry, 1930].

I. The histamine-histaminase reaction.

The results of preliminary work on the rate of the reaction indicated that (1) after mixture of certain proportions of histamine and enzyme, there was, previous to actual inactivation, a phase during which no change in histamine, as shown by the depressor activity, occurred; (2) when the quantity of histamine used was constant, the total amount of amine destroyed depended on the concentration of enzyme; (3) the amount of histamine inactivated by a constant amount of enzyme was not markedly affected by the histamine

concentration. The second and third observations have been confirmed by Gebauer-Fuelnegg and Alt.

More accurate measurements of the rate of inactivation have been made in an attempt to elucidate the nature of the changes occurring. The general method was as follows: a solution of histaminase prepared by extracting kidney powder with phosphate buffer was added to a solution of histamine acid phosphate in phosphate buffer at p_H 7·2. Toluene was used as a preservative and the mixture was kept, with continuous agitation by means of a stirrer or a current of gas, at 38° in a water-bath. Samples for histamine determinations were removed at intervals through a siphon dipping beneath the toluene.

In the first series the proportion of enzyme to histamine was such that the amine would be inactivated in about 24 hours. A histaminase solution was prepared by extracting 30 g. of ox kidney powder with 270 cc. phosphate buffer, p_H 7·2, for one hour with continuous agitation. After filtration 75 cc. were added to phosphate buffer, p_H 7·2, containing 30 mg. histamine acid phosphate (10 mg. base) the volume was made up to 500 cc. with buffer and 50 cc. of toluene were added. There was a definite preliminary period of 5 hours during which no change in the depressor activity of the solution could be detected. The values secured during the second, inactivating phase lie fairly well on a smooth curve (Fig. 1). On semilogarithmically ruled paper the inactivation phase is represented by a straight line. The values of k in the equation $k = \frac{1}{t} \log \frac{a}{a-x}$, where a = initial amount of histamine and x = amount inactivated, have been calculated. When this is done, using for t values from the initial admixture, k is not a constant. If the values of t be corrected by subtracting the length of the lag phase from the elapsed time, k is constant (Table I). As judged from these values for the velocity constant, the reaction is unimolecular.

Table I. *Velocity constant of histamine-histaminase reaction.*

t	$t_1 = t - 5$	a	$a-x$	$k = \frac{1}{t_1} \log \frac{a}{a-x}$
6 hrs.	1	10 mg.	8·5 mg.	0·071
8 ,,	3	10 ,,	6·4 ,,	0·065
10 ,,	5	10 ,,	4·7 ,,	0·066
12 ,,	7	10 ,,	3·5 ,,	0·065
14 ,,	9	10 ,,	2·5 ,,	0·067
16 ,,	11	10 ,,	1·6 ,,	0·072

Determinations of a similar nature have been carried out with varying proportions of enzyme. These all showed the two phases. The duration of the lag phase was inversely proportional to the concentration of histaminase, and the rate of the inactivation phase was directly proportional to the concentration. In each case the rate of the second phase was logarithmic.

Earlier work had shown that in the absence of oxygen and when nitrogen or hydrogen was present almost exclusively the histamine-histaminase reaction

did not occur. Determinations of the rate, similar to those previously made, were carried out with large amounts of oxygen bubbling through the mixture. In these the length of the lag phase was one-half that found without the stream of oxygen but with the same concentration of enzyme and of histamine. No great change was observed in the second phase. In another experiment three sets of measurements were made on the same day using portions from one solution of enzyme in each set. Through one mixture air was bubbled, through the second oxygen and through the third nitrogen. The results are shown in

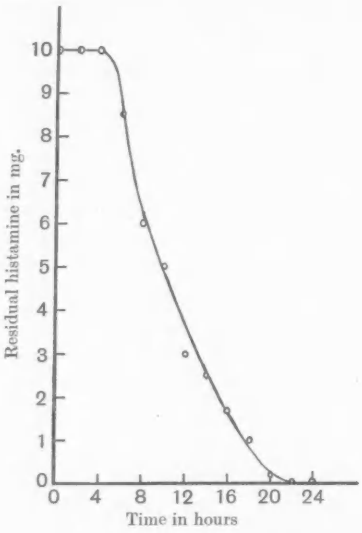

Fig. 1. Rate of inactivation of histamine.

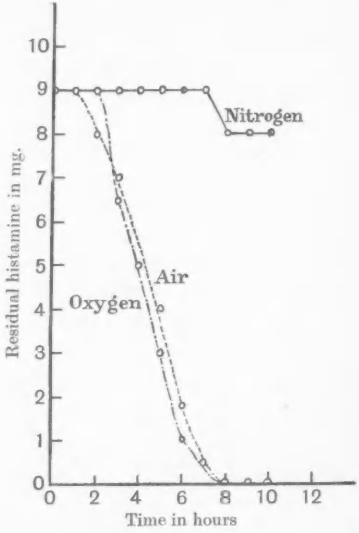

Fig. 2. Effect of nitrogen and oxygen upon histaminase.

Fig. 2. The rates for air and oxygen are practically identical but nitrogen definitely slowed the reaction, scarcely any inactivation occurring.

It had previously been found that low concentrations of potassium cyanide prevented the inactivation of histamine. It seemed advisable to determine whether the addition of cyanide to a previously cyanide-free mixture in which inactivation was progressing would stop any further action of the enzyme. For this three flasks were used. Each flask contained 75 cc. histaminase solution (the filtrate secured after extraction for one hour of 60 g. of ox kidney powder with 540 cc. phosphate buffer), 30 mg. histamine acid phosphate (10 mg. base), sufficient buffer of p_H 7·2 to make a volume of 500 cc. and 50 cc. toluene. The results are shown in Fig. 3. In the first case 0·002 *M* potassium cyanide was initially present and no inactivation occurred. In the second case an equal amount of cyanide was added when 25 % destruction had taken

place, and it is evident that further inactivation was inhibited. The third curve demonstrates that this particular enzyme preparation was acting in the customary manner. In view of the results of Gebauer-Fuelnegg and Alt [1932] which suggested incomplete inhibition by cyanide it is interesting that we have repeatedly found cyanide to prevent the initiation of the inactivation and to cause its cessation when added at an intermediate stage.

Since cyanide had an inhibitory effect upon the enzyme it seemed desirable to investigate the action of sodium pyrophosphate. In a concentration of 0·05 M at p_H 7·2 this salt has no effect upon the histamine-histaminase reaction. As has been suggested by Gebauer-Fuelnegg and Alt, iron is probably excluded as an active agent, while copper may be a factor.

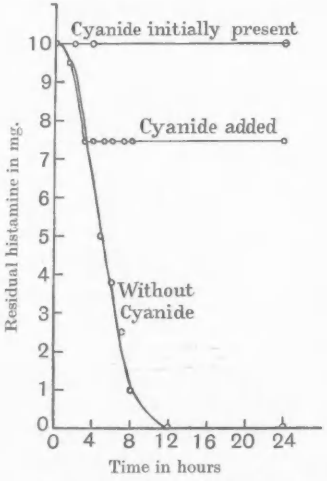
Cyanide initially present
Cyanide added
Without Cyanide
Residual histamine in mg.
Time in hours
0 4 8 12 16 20 24
0 1 2 3 4 5 6 7 8 9 10

Fig. 3. Effect of cyanide upon histaminase.

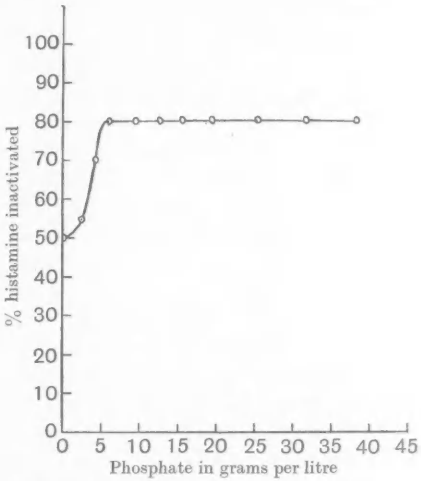

Fig. 4. Effect of concentration of phosphate upon histaminase.

Of the various ions investigated, phosphate alone has a definitely accelerating effect upon the action of histaminase. This has been studied quantitatively and the results are given in Fig. 4. Phosphate is not essential for the reaction, which proceeds in the absence of this ion, but the amount of histamine inactivated by a constant amount of enzyme in a constant time is increased when phosphate is present. There is no definite concentration of phosphate which may be regarded as optimal, rather there is an optimal range. The amount of phosphate present in our routine measurements of histaminase action lies within this range.

The changes produced in histamine by histaminase are of considerable interest. In the case of the amine, tyramine, Ewins and Laidlaw [1910] found that, after removal of the amino-group from the side-chain, the remnant was

oxidised to the alcohol and then to the acid. Guggenheim and Loeffler [1916] showed that similar reactions occurred in the cases of phenylethylamine, indolethylamine, and *iso*amylethylamine. They were unable to isolate iminazolyl acetic acid after inactivation of histamine. In view of the destruction of the iminazole ring previously reported [Best and McHenry, 1930], the failure of Guggenheim and Loeffler in the case of histamine is explicable. In experiments with tyramine and tyramine oxidase conducted *in vitro*, Hare [1928] found that deamination occurred but that only half the nitrogen present in the tyramine molecule could be accounted for as ammonia. In an extension of this work [Bernheim, 1931] the same author showed that the course of oxidation of tyramine varied under different conditions. Under certain conditions, *p*-hydroxyphenylacetic acid was produced, and in this case there was complete deamination of tyramine. A quantitative study of ammonia production was not completed. To gain some information on the changes produced in histamine, measurements have been made of ammonia produced during the reaction.

To determine the amount of ammonia various methods have been tried. The procedure found most useful was, after the customary incubation of a mixture of kidney powder and histamine in phosphate buffer, to filter this mixture through Whatman No. 41 paper. The ammonia content of a 10 cc. aliquot portion of the filtrate was measured, using the Van Slyke and Cullen aeration apparatus. In nearly every case duplicates were aerated and afterwards the ammonia from one was determined by titration and from the other by nesslerisation.

In all the ammonia determinations the source of histaminase was a potent hog kidney powder, of which 50 mg. would inactivate 2 mg. histamine in 24 hours at 38°. For each determination 1 g. of this powder and 120 mg. histamine acid phosphate in 100 cc. phosphate buffer were employed. Initially the incubation was carried out in buffer solutions at p_H 7·2 but later two other hydrogen ion concentrations were used. The amount of residual histamine in each case was measured in the customary manner. In place of toluene, "merthiolate"[1] in 0·01 % concentration was used as preservative.

The first series was designed to measure the ammonia produced during the histamine-histaminase reaction under the conditions normally used by us: p_H 7·2, 24 hours' incubation at 38°. At first controls were flasks in which the enzyme had been inactivated by boiling and in these some ammonia was found. The difference between the ammonia content of the active enzyme flask and the boiled control was not of a simple molar nature and it was found that this type of control was not satisfactory. Although there is no histamine in the kidney powder, incubation of the powder in buffer without added histamine gives a demonstrable amount of ammonia, produced from the powder during the incubation, and appreciably greater than the amount found in a boiled control. Consequently similar mixtures of unheated powder and buffer with preservative were used in all subsequent work as controls. Table II, A gives results secured at p_H 7·2.

[1] The Eli Lilly Co.

Table II. *Ammonia formation.*

Histamine inactivated mg.	Total possible ammonia from histamine mg.	Ammonia found (mg.) Titration	Nessler	Average	Ammonia produced from histamine mg.	Ammonia found in % of theoretical
A. At p_H 7·2:						
2	0·90	0·61	0·67	0·64	0·29	32
0	0	0·32	0·37	0·35		
2	0·90	0·59	0·61	0·60	0·29	32
0	0	0·29	0·33	0·31		
3	1·35	0·79	0·78	0·79	0·46	34
0	0	0·31	0·35	0·33		
2	0·90	0·57	0·65	0·61	0·32	35
0	0	0·27	0·31	0·29		
4·0	1·80	0·90			0·56	31
0	0	0·34				
1·5	0·68		0·44		0·19	28
0	0		0·25			
					Average =	32
B. At p_H 5·9:						
1·6	0·72	0·39	0·37	0·38	0·15	21
0	0	0·23	0·22	0·23		
1·6	0·72	0·33	0·29	0·31	0·14	19
0	0	0·17	0·16	0·17		
1·8	0·81	0·55	0·47	0·51	0·15	19
0	0	0·36	0·35	0·36		
1·4	0·63	0·29	0·27	0·28	0·12	19
0	0	0·17	0·15	0·16		
2·0	0·90	0·53	0·44	0·49	0·15	17
0	0	0·35	0·33	0·34		
1·6	0·72	0·34	0·31	0·33	0·14	19
0	0	0·19	0·18	0·19		
2·0	0·90	0·67	0·54	0·61	0·22	24
	0	0·40	0·38	0·39		
1·8	0·81	0·35	0·35	0·35	0·15	19
0	0	0·19	0·21	0·20		
2·0	0·90	0·64	0·59	0·62	0·20	22
0	0	0·42	0·42	0·42		
					Average =	20
C. At p_H 8·8:						
3·6	1·62	0·70	0·65	0·68	0·47	29
0	0	0·21	0·21	0·21		
4·0	1·8	0·91			0·49	27
0	0	0·42				
4·0	1·8	0·74			0·53	29
0	0	0·21				
4·0	1·8	0·91			0·49	27
0	0	0·42				
2·0	0·90	0·40	0·41	0·41	0·23	26
0	0	0·17	0·19	0·18		
2·0	0·90	0·48	0·43	0·46	0·27	30
0	0	0·20	0·18	0·19		
3·5	1·61	0·65	0·66	0·66	0·46	29
0	0	0·19	0·21	0·20		
3·1	1·40	0·62	0·55	0·59	0·41	29
0	0	0·19	0·17	0·18		
					Average =	28

The average amount of ammonia found was 32 % of the quantity which could be produced if all three nitrogen atoms in the histamine molecule had been liberated as ammonia. Under these conditions, then, only one nitrogen, probably from the side-chain, is liberated as ammonia.

It was imperative to make sure that the procedures used were giving reliable results. Aeration of ammonium sulphate solution and of kidney solution containing added ammonium sulphate showed that the analytical method was accurate. Moreover recovery of ammonium sulphate which had been added to digests before incubation indicated that ammonia was not lost during incubation. It is reasonable to conclude that the amount of ammonia measured is the total amount produced during the histamine-histaminase reaction under the experimental conditions.

Determinations were made of ammonia production at two other hydrogen ion concentrations, all other conditions being as similar as possible to those used at p_H 7·2. The results are given in Table II, B and C. As before, control experiments showed that ammonia was quantitatively recovered. While the ratio of ammonia produced to histamine inactivated at p_H 8·8 was slightly lower than at p_H 7·2 the difference is too small to be significant and again one molecule of ammonia was formed per molecule of histamine inactivated. At the other p_H, 5·9, this is obviously not true, the amount of ammonia being much less than at 7·2 and at 8·8. The explanation of this is not obvious at present. Since the amount of ammonia found is less than would be expected if all the inactivated histamine had been deaminised it is reasonable to conclude that a mechanism other than deaminisation is operative. Previous work in this laboratory had shown that under certain conditions there was a rupture of the iminazole ring. In the histamine-histaminase reaction both deaminisation and a change in the nucleus may take place and the relative intensities of these two processes may not be the same at different hydrogen ion concentrations.

The rate of ammonia formation in comparison with the rate of loss of physiological activity at p_H 7·2 was measured using a procedure similar to that previously described for velocity measurements. At definite time intervals portions were removed from the incubated mixture and measurements made of histamine and of ammonia present. The control again consisted of a kidney extract without added histamine and the ammonia from histamine was calculated as the difference between that of the histamine mixture and of the control. The results are given in Fig. 5. Calculations were made of the ammonia theoretically possible at each interval, assuming that one molecule had been produced for each molecule of histamine inactivated. Between the curve for these values and that for experimental results there is good agreement. At this p_H, then, the rate of ammonia production is in accordance with the velocity of histamine inactivation.

It seemed possible that urea might be formed during the reaction by a cleavage of the iminazole nucleus. Urea determinations in inactivated mixtures were made by the urease method and it was found that no compound which

could be decomposed by urease to give ammonia had been formed. Urea added to the solution was quantitatively determined by the method, indicating that the urease had not been inhibited by a constituent of the mixture. It may be that a urea derivative which could not be split by urease is formed but certainly urea itself is not produced during the histamine-histaminase reaction.

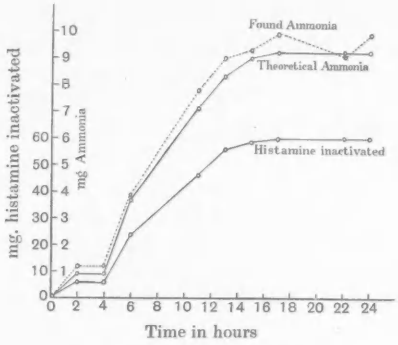
Found Ammonia
Theoretical Ammonia
Histamine inactivated
mg. histamine inactivated
mg Ammonia
Time in hours
0 2 4 6 8 10 12 14 16 18 20 22 24
10 9 8 7 6 5 4 3 2 1
60 50 40 30 20 10 0

Fig. 5. Rate of ammonia formation.

II. Attempts to purify histaminase.

Since it is essential to prepare from kidney powder a solution containing the enzyme, conditions for increasing the yield on extraction have been studied. Water gives a potent extract, but the yield is greater when phosphate buffer at p_H 7·0–7·2 is used. Solutions thus secured are unstable but may be stabilised by the addition of glycerol, sufficient to give 15 % concentration. Extracts prepared at 38° are more potent than those at room temperature and these in turn are more potent than those secured by extraction in the cold. Solutions prepared by extracting at $p_H < 5$ are inactive and are not activated by neutralisation after extraction. A slightly increased yield is secured by extraction at p_H 8 but the alkaline solution filters very slowly. Larger yields are given by fairly dilute extraction, and we have found a suitable proportion to be 18 parts of liquid to one part of kidney powder. One such extraction at 38° gives about 60 % of the total potency of the powder, and a further 20 % results from a second extraction. The combined extracts may be concentrated *in vacuo* to one third the original volume without loss in activity, provided that the temperature of the bath surrounding the distillation flask be kept below 50°.

Three methods for the precipitation of histaminase have been investigated. If the concentrated, aqueous solution be poured slowly, with stirring, into a large volume of acetone at room temperature, a precipitate is secured which contains about 70 % of the total activity. This precipitate, after decantation of the acetone, dries quickly in air, and is relatively stable. If alcohol, ether, or mixtures of alcohol and ether are used in place of acetone the precipitate

produced is inactive. Precipitates which settle out from the original aqueous solution after adjustment to p_H 5 are also inactive. Four salts have been tried as precipitating agents. Sodium chloride in 25 % concentration at room temperature and sodium sulphate in various concentrations up to saturation at room temperature do not precipitate any active histaminase, despite the fact that the latter salt gives a heavy precipitate. When a solution is saturated with ammonium magnesium sulphate, histaminase is completely precipitated. This precipitate may be dried in a vacuum oven at temperatures below 50° without decrease in potency, and the dry powder is fairly stable, retaining its activity for at least a month.

The ammonium sulphate precipitate contains much sulphate, but a precipitate with low sulphate content is secured by using 80 to 90 % saturation. At this concentration of ammonium sulphate histaminase is completely precipitated. The conventional method for removing salt by dialysis results in a loss of about 70 % of the enzymic activity. A procedure developed by Brieger and Cohn [1893], and suggested to us by Dr P. J. Moloney, for the separation of tetanus antitoxin from ammonium sulphate has proved useful. This is based on differences in specific gravity. The salt-protein mixture is rubbed up with chloroform, more chloroform added and the whole centrifuged. Two solid layers separate: at the top proteins, and at the bottom the salt. This procedure gives good results with the histaminase precipitate. The top layer is poured on a hard filter-paper and allowed to dry in air. The resulting fine, tan-coloured powder contains over 70 % of the potency. Its activity is about ten times that of the original kidney powder, and it is relatively stable. While this procedure is an improvement on previous methods, it has not yet been possible to prepare histaminase in highly concentrated form.

III. Distribution of histaminase in the kidneys of various species.

It had previously been shown that, in the dog, histaminase was present in largest amounts in the kidney and in the intestinal mucosa. It seemed desirable to determine the variation in kidney histaminase among several species of animals. For this, where possible, normal animals were placed under ether anaesthesia and the kidneys removed. In some cases tissues were secured from abattoirs as soon as possible after the death of the animal. The human kidneys were autopsy specimens. In larger animals each kidney was tested separately while in smaller animals it was necessary to combine the kidneys from several animals to secure adequate amounts. After finely mincing the tissue, portions were weighed and transferred to 500 cc. Erlenmeyer flasks. In every case 5 g. of tissue were used for each determination. To each flask were added 100 cc. phosphate buffer, p_H 7·2, the required amount of histamine acid phosphate, and 15 cc. toluene. The mixture was incubated for 24 hours at 38° and the residual histamine determined by physiological assay. With each set controls were run, in which the enzyme had been inactivated by heating to boiling.

These controls acted as checks upon the total amount of histamine present, both added and contained in the tissue originally. The amount of histamine inactivated by a given amount of enzyme varies somewhat with the amount of histamine initially present. In each case determinations with varying amounts of histamine were made. The maximum inactivation was secured when 150 mg. of histamine acid phosphate (50 mg. histamine) were used with 5 g. kidney. The results in Table III are based on this proportion, and are averages of determinations made on four or more members of each species.

Table III. *Histamine inactivated by kidneys from various species of animals.*

Animal	Mg. histamine inactivated by 1 g. kidney
Rat	0
Guinea-pig	0
Rabbit	1·6
Dog	1·9
Cat	2·0
Ox	1·6
Hog	3·6
Horse	2·7
Sheep	3·2
Monkey	2·4
Human	0–1·6
Chicken	0

There is no apparent correlation between these results and available figures for histamine distribution in various species (Raxlen, unpublished data), or for sensitivity to histamine [Schmidt and Stähelin, 1929]. Among the individuals in any one species there is considerable variation, as shown in Table IV.

Table IV. *Kidney histaminase variation in members of a species*

Animal	Mg. histamine inactivated by 1 g. kidney	Average
Sheep	4·0, 2·8, 3·2, 4·0, 2·0, 3·2	3·2
Ox	1·2, 2·0, 1·2, 2·0, 1·2, 2·0	1·6
Hog	4·0, 2·8, 3·2, 3·2, 5·2, 3·2	3·6

As far as could be ascertained, the kidneys used in these distribution studies were normal. It would be interesting to investigate the histaminase content of kidneys in various pathological conditions. Preliminary experiments, using cats and dogs, were made in an attempt to alter the histaminase content of the kidney by extended administration of histamine subcutaneously, or by holding the animal in histamine shock. The results have been inconclusive. There has been no marked depletion in kidney histaminase after shock nor has it been found possible to increase kidney histaminase in dogs by giving histamine in small daily doses over a long period.

SUMMARY.

Previous to the actual inactivation of histamine by histaminase there is a lag period, the length of which varies inversely with the enzyme concentration. The rate of the inactivation is logarithmic and, as judged from values secured for the velocity constant, the reaction is unimolecular. The inactivation

is accelerated in an atmosphere containing oxygen and is inhibited in an atmosphere deficient in oxygen. Small amounts of potassium cyanide not only prevent the initiation of the reaction but cause its cessation when added at an intermediate point. Sodium pyrophosphate has no inhibitory effect and the phosphate ion augments the reaction. Measurements of the ammonia production from histamine show that at p_H 7·2 and 8·8 histaminase causes the liberation of one nitrogen atom, presumably that of the side-chain, as ammonia. At p_H 5·9 less ammonia is produced. While attempts at purification have not as yet yielded a very concentrated preparation, information on a number of the properties of histaminase has been obtained. An investigation of kidney-histaminase in various species has shown that it is not demonstrable in the rat, guinea-pig and chicken, and is present in varying amounts in several mammals. Of the tissues so far investigated, hog kidney is the best source of histaminase and a method has been described for the preparation from this tissue of a stable powder which may be used in the study of histaminase.

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